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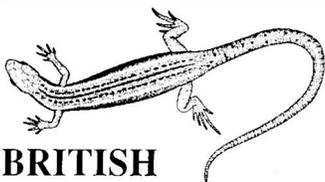
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FRONT COVER: Grass snake, *Natrix natrix* (B. Lewis)

CLOSE ENCOUNTERS OF THE WORST KIND: PATTERNS OF INJURY IN A POPULATION OF GRASS SNAKES (*NATRIX NATRIX*)

PATRICK T. GREGORY AND LEIGH ANNE ISAAC

Department of Biology, University of Victoria, Victoria, BC, Canada

Injuries of various types are widespread in animals and presumably have implications at the population level (e.g. reduced future survivorship). We studied patterns of injury acquisition in a population of grass snakes (*Natrix natrix*) in south-eastern England. Injuries suffered by grass snakes were of various types, including broken bones, assorted scars and wounds, and tail loss. What causes such injuries is unknown, but predators seem most likely. We predicted that the probability of having an injury would be higher for larger snakes, for several reasons (e.g. larger snakes are older and thus have had more opportunity to be injured). We also predicted that injury rates would be higher in females because, when gravid, they are expected to bask in the open more than other snakes. Our data strongly supported the first of these predictions, but not the second. Males had significantly higher injury rates than females of the same body size. However, because males grow more slowly and mature at a smaller body size than females, higher injury rates of males might simply reflect their smaller size at a given age. Even if age plays a role in influencing acquisition of injuries, other, more directly size-related factors also might be important. Two possibilities are that small snakes might be less likely to survive an injury or that small snakes spend more time hidden and so are less likely to encounter large predators. We lack data on the first of these, but data on sizes of snakes found under cover versus those found in the open are consistent with the second. Studies of injury rates in snakes need to move beyond the descriptive stage and begin to test the broader consequences of injuries.

Key words: Colubridae, injuries, natricine, snake, predation, southern England

INTRODUCTION

Close encounters with predators, intraspecific aggressors, or other misadventure are common in the lives of animals. Although such encounters can result in death, they sometimes result in nonlethal injury. Injuries of various kinds, including loss of body parts, have been reported for diverse taxa (Vermeij, 1982; Harris, 1989), among them centipedes (Fründ *et al.*, 1997), crustaceans (Rigaud & Juchault, 1995; Dyrinda, 1998; Plaistow *et al.*, 2003), spiders (Taylor & Jackson, 2003), echinoderms (Aronson, 1987; Baumiller & Gahn, 2004), snails (Warren, 1985), fish (Reimchen, 1988), amphibians (Maiorana, 1977; Pflingsten, 1990; Gray *et al.*, 2002), reptiles (Schoener & Schoener, 1980; Willis *et al.*, 1993; Meek, 1989), birds (Randall *et al.*, 1988), and mammals (Lidicker, 1979; Rose, 1979; Shargal *et al.*, 1999; Macdonald *et al.*, 2004).

What are the broader, population-level, consequences of injury? Whether injury rates can be used to infer predation rates is debatable (Jaksic & Greene, 1984; Greene, 1988; but see Baumiller & Gahn, 2004), especially in the absence of survival and other data (Schoener, 1979). Rather, incidence of injury might be more indicative of predator inefficiency (Reimchen, 1988; Mushinsky & Miller, 1993). However, injuries have possible costs in terms of future survival and reproduction, and, in some cases, might act to regulate population size (Harris, 1989). Injured animals may fare

more poorly in intrasexual conflicts (Taylor & Jackson, 2003). Failed predation, as evidenced by injury, is a necessary condition for the evolution of antipredator characteristics (Vermeij, 1982). Conversely, injured and non-injured animals, which differ morphologically in some species, might reflect adaptations for surviving injury vs. avoiding injury, respectively (Seligmann *et al.*, 2003). Wounds are also points of entry for infectious micro-organisms (e.g. Dyrinda, 1998) and might influence selection for investment in immune defences (Plaistow *et al.*, 2003). In arthropods, wounds represent a possible means of horizontal transfer of the feminizing bacterium, *Wolbachia*, which in turn leads to sex-ratio distortion (Rigaud & Juchault, 1995). Thus, data on injury rates have potential value for revealing population-level phenomena.

In squamate reptiles, most studies of patterns of injury have been done on lizards, largely because many species of lizards autotomize the tail in response to attempted predation on them (review in Arnold, 1988). Although shedding the tail and escaping from a predator have immediate survival value, tail loss also has costs in many cases and most autotomizing lizards regenerate the tail (Arnold, 1988; see also plethodontid salamanders – Maiorana, 1977).

Studies of injuries in snakes are at a less analytical, more descriptive stage (but see Willis *et al.*, 1993; Slowinski and Savage, 1995). However, numerous authors have reported injuries, including tail loss, in snakes (review in Greene, 1988). Taxa for which injury data are available include uropeltids (Greene, 1973), erycine boas (Greene, 1973; Hoyer & Stewart,

Correspondence: P. Gregory, Department of Biology, University of Victoria, PO Box 3020, Victoria, BC, Canada, V8W 3N5. *E-mail:* viper@uvic.ca

2000a,b), colubrids (Fitch, 1963; Leavesley, 1987; Mendelson, 1992; Slowinski & Savage, 1995; Capula *et al.*, 2000), and viperids (Macartney, 1985). Among colubrids, much work has focused on the natricine genera *Nerodia* (Mushinsky & Miller, 1993; Fitch, 1999) and *Thamnophis* (Willis *et al.*, 1993; Fitch, 1999, 2003), and several other studies of these genera have incidentally noted injuries (e.g. Diener, 1957; Preston, 1970; King, 1987). Although snakes do not regenerate their tails once lost (Greene, 1988), some species may practise a form of caudal autotomy (Broadley, 1987; Greene, 1988; Cooper & Alfieri, 1993; Fitch, 1963, 1999; Akani *et al.*, 2002), which Slowinski & Savage (1995) argue is more correctly called 'pseudautotomy'. Frequency of tail loss is higher in species with relatively long tails (Kaufman & Gibbons, 1975) and species with specialized pseudautotomy may experience multiple tail breaks through life (Slowinski & Savage, 1995). Loss of part of the tail can lead to reduced mating success in males (Shine *et al.*, 1999), but apparently has little effect on locomotory speed (Jayne & Bennett, 1989). Potential consequences of other kinds of injuries have not been investigated.

In this study, we documented patterns of injury in a population of the natricine grass snake (*Natrix natrix*) in southern England. In addition to recording the incidence of injury, we tested two predictions. First, we predicted that the relative frequency of injury should be higher in older (and therefore larger) snakes because, all else being equal, older animals should have a higher probability of having acquired an injury sometime in their life (Willis *et al.*, 1993). An alternative, but not mutually exclusive, explanation for the higher occurrence of injuries in larger snakes is that larger snakes simply are more likely to withstand and survive a predation attempt than are small snakes (Willis *et al.*, 1993; Mushinsky & Miller, 1993). A third possible explanation, also not mutually exclusive with the others, is that because they are more vulnerable, smaller snakes spend more time hiding under cover, rather than exposing themselves to larger predators that forage in the open. One important distinction between these three hypotheses, however, is that in the first one, body size is merely a surrogate for age, whereas, in the other two, it is the variable of primary interest. Second, we predicted that female grass snakes would have higher injury rates than males because (1) when gravid, females spend more time basking in the open than other adults (Madsen, 1987), exposing themselves to increased risk of potential injury; and (2) females reach larger body sizes than males (Madsen, 1983; Gregory, 2004), so factors outlined above should apply.

MATERIALS AND METHODS

We collected the data for this study at Fordwich, near Canterbury, Kent in south-eastern England. The study site is centred around a series of water-filled gravel quarry pits on either side of the River Stour. We captured snakes by hand, mainly in the open but

occasionally under cover objects, during 3-week to 4-month visits to the site in each year from 1999–2003, inclusive. We measured several variables on each snake at its capture site and released it within 10–20 min of capture. These included snout-vent length (SVL), tail length (TL), sex, and presence of injury. We did not begin to record TL until part-way through the 2000 sample. We did not tag animals, but identified them individually by patterns of anterior ventral markings, recorded either by drawings or photographs. In addition to field-caught snakes, we collected similar data from a few hatchlings obtained from eggs incubated in the laboratory; these eggs were laid by females from the Fordwich field site.

We analyzed the data using SAS 8.0 and a nominal rejection level of $\alpha=0.05$. To maintain independence of data, we excluded all recaptures from analyses and used only original captures. We used logistic regression to test the influence of SVL and sex on occurrence of injury (binomial variable: presence/absence) and contingency tables to compare frequencies of categorical variables.

RESULTS

We obtained data from 87 female and 93 male grass snakes. Of these, 21 were captive-hatched (12 females, 9 males). The two sexes differed significantly in SVL (Kruskal-Wallis $\chi^2_1=32.55$, $P<0.0001$), females reaching much greater maximum and median SVLs than males (960 mm vs 740, 715 vs. 562, respectively; hatchlings included).

No hatchlings showed any evidence of physical injury. Thus, we assumed that snakes do not commonly hatch with deformities that resemble injuries and that injuries therefore are acquired later in life.

Of 159 field-caught snakes for which we recorded the presence or absence of injuries, 71 had injuries of various types. Some (24) had lost part of their tail, ranging from just the tip to a larger amount that left a pronounced stump. Most snakes that had lost a large part of their tails (expressed as deviation from TL-SVL relationship for snakes with intact tails) were large (Fig. 1). Fifty-seven snakes, including 11 of those with stumped tails, had other kinds of injuries, some minor and others more serious, sometimes multiple, on various parts of the body from head to tail, inclusive. These other injuries included assorted scars, some old and others fresh, but some snakes showed evidence of having had broken bones (now healed), either spine or ribs.

We divided injuries into two main categories, tail loss and others, and found no association between their occurrence in field-caught snakes ($\chi^2_1=1.17$, $P=0.28$); that is, snakes with stumped tails and those with intact tails were equally likely to also have other injuries. There also was no overall association between sex and the frequency of stumped tails, other injuries, or all injuries combined (latter: $\chi^2_1=0.005$, $P=0.94$). These conclusions were not changed by including hatchlings in the analyses. However, distinct patterns emerged when

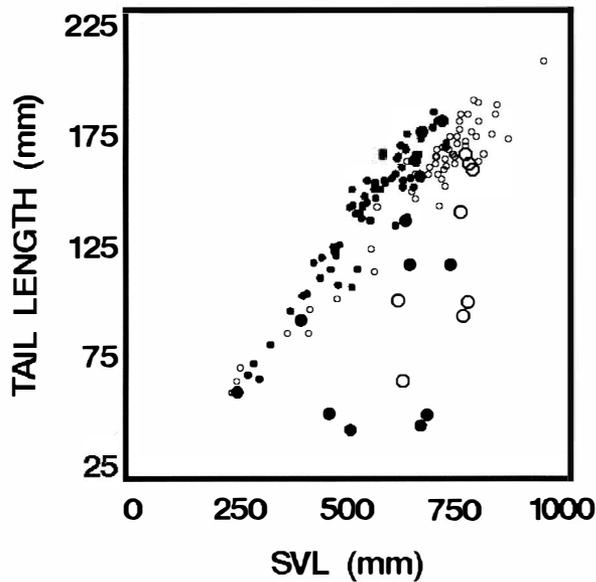


FIG. 1. Tail length vs SVL for grass snakes with intact (small symbols) tails and stumped (large symbols) tails. Open circles, females; closed circles, males. Deviation of large points from small points at same SVL indicates approximate amount of tail lost. Sample ($N=141$) includes field-caught snakes (original captures only), but not captive-born hatchlings.

we considered the effect of body size using logistic regression.

Because we had no reason to expect that captive-born hatchlings would differ from wild-born ones in injury status, we included the former in logistic regressions. Furthermore, although our conclusions were not affected by leaving hatchlings out, the fit to the model was substantially improved by including them (Hosmer & Lemeshow goodness-of-fit nonsignificant), which also slightly increased the range of SVLs in the analysis.

The regression of $P(\text{injury})$ on SVL was highly significant (Wald's $\chi^2_1=21.86$, $P<0.0001$), with probability of having an injury increasing with SVL (Fig. 2A). Adding sex as a factor improved the model (AIC = 208.40 vs 214.18 for SVL alone) and showed that males had a significantly higher injury rate than females at a given SVL (Fig. 2B; Wald's $\chi^2_1=6.99$, $P=0.008$), with the effect of SVL also remaining highly significant (Wald's $\chi^2_1=20.79$, $P<0.0001$).

As a preliminary test of whether smaller snakes spend more time under cover and are therefore less likely to be exposed to predators that forage in the open, we compared the sizes of snakes caught under cover objects to those captured in the open. Although only nine of 159 field-caught snakes were found under cover objects, they were significantly smaller than those caught in the open (medians 410 mm SVL vs 656.5; Kruskal-Wallis $\chi^2_1=18.21$, $P<0.0001$).

DISCUSSION

The results of this study provide strong support for our prediction that the occurrence of injury increases

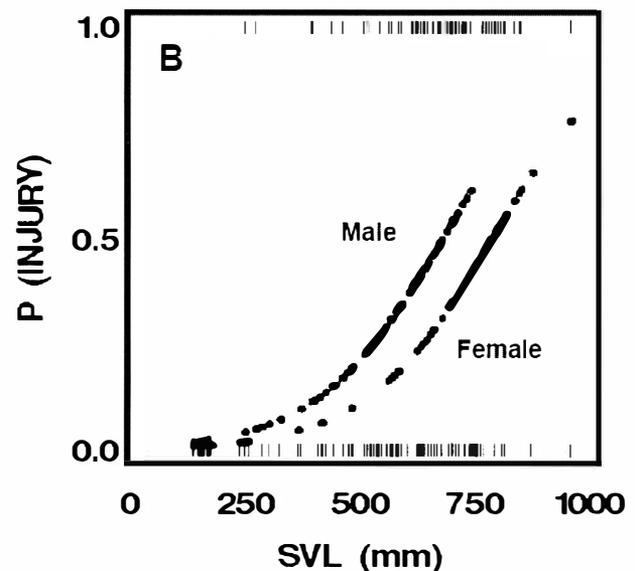
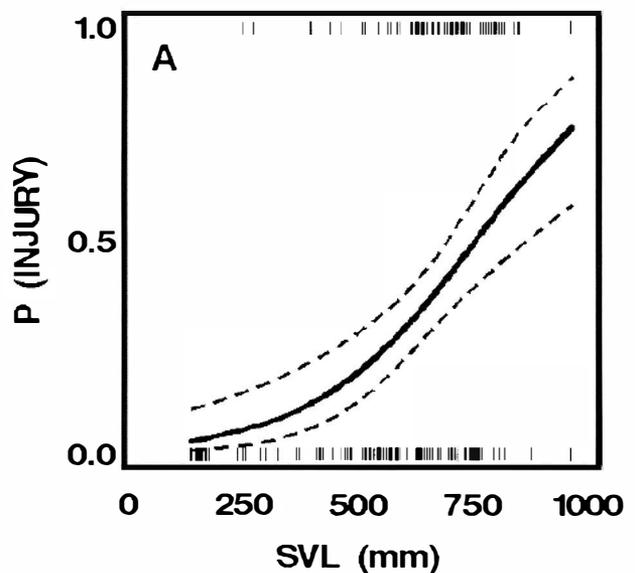


FIG. 2. Logistic regressions of probability of having acquired an injury vs SVL for grass snakes. A, both sexes pooled (vertical lines at top = injured snakes, vertical lines at bottom = uninjured snakes, $N=180$, including both field-caught snakes and captive-born hatchlings; solid line represents predicted values from logistic regression of injury/no injury against SVL; dotted lines represent 95% confidence limits on predicted values). B, sex treated as an independent variable with two levels (predicted values only shown; top curve – males, lower curve – females; regression lines for each sex analyzed separately are slightly different, but still show the same relative positions).

with body size, consistent with most other studies of snakes, including grass snakes (Borczyk, 2004). As we did not distinguish between old and recent injuries, it is possible that we may have underestimated the actual incidence of injury because we missed old injuries that had healed sufficiently to be no longer obvious. However, it is unlikely that this would have changed our overall conclusions. Mushinsky & Miller (1993) fo-

cused only on recent wounds and still found the same trend for increasing occurrence of injury with increasing body size. Most studies of tail breaks in snakes also have shown that the incidence of this kind of injury, which is permanent, is higher in larger snakes (e.g. Mendelson, 1992; Fitch, 1963, 1999, 2003). However, Willis *et al.* (1993) found no evidence of size-based variation in tail breakage in one of the three species that they studied.

In contrast, our prediction of higher injury rates in female grass snakes, compared to males, was not upheld. Data on other natricine species with female-biased sexual size dimorphism (SSD) apparently support this prediction, in general. For example, Fitch (2003) found that, among adult *Thamnophis sirtalis*, females had broken tails significantly more often than males. Fitch (1999) also compared tail injuries between the sexes in water snakes, *Nerodia sipedon*, by probable-age groups, and again showed a higher incidence of injury in females. Willis *et al.* (1993) also concluded that, overall, female *Thamnophis* had higher rates of tail breakage than males, but inspection of their Fig. 1 shows that the incidence of this injury was higher in adult males than females in *T. sirtalis*, similar to our findings. Mushinsky & Miller (1993) found no difference in the occurrence of fresh injuries on males and females of six species of natricines (five *Nerodia*, one *Regina*), but did not make any corrections for size or age variation. Such correction seems crucial for making comparisons between the sexes in species with pronounced SSD. Capula *et al.* (2000) found no difference in frequency of tail breakage in the colubrine *Coluber viridiflavus*, a species with male-biased SSD, but they also did not adjust for age or size differences. In the dipsadine *Coniophanes fissidens*, which lacks SSD, there were no consistent inter-sexual differences in tail-breakage rate among populations and no difference between the sexes overall (Mendelson, 1992).

We hypothesized that females would experience higher rates of injury partly because, when gravid, they are reported to bask more than other snakes (Madsen, 1987), presumably for thermoregulatory purposes. For example, Akani *et al.* (2002) found that gravid females of the African snake, *Psammophis philipsii*, had a significantly higher frequency of tail loss than nongravid snakes (which included both males and females). However, Isaac & Gregory (2004) found no evidence that gravid female grass snakes in an outdoor enclosure thermoregulated more precisely or maintained higher body temperatures than nongravid females. Although we have not tested male snakes, this might help explain why female grass snakes at our study site did not have a higher injury rate than males.

In fact, after adjusting for body size, we found that male snakes had a higher frequency of injury than females. Although we lack sufficient data to construct growth curves for grass snakes at Fordwich, this apparent difference could be an artefact of intersexual differences in growth rates. Growth rate in this species is phenotypically plastic and varies geographically, but fe-

males typically attain larger maximum sizes than males (Madsen & Shine, 1993a; Luiselli *et al.*, 1997). Furthermore, females also may grow faster than males (Madsen, 1983; but see Luiselli *et al.*, 1997). If so, the higher injury rate of males of a given SVL might only reflect the fact that they are older than similar-sized females. This can be tested directly with data on age (e.g. Wayne & Gregory, 1998; Wayne, 1999) and, if confirmed, would suggest that age *per se* is an important factor influencing the likelihood of having sustained an injury.

Age may be important, but what about other, size-related factors that might affect incidence of injury? For example, do smaller snakes have fewer injuries simply because they are less likely to survive an attack from a predator, either immediately or over the short term? Willis *et al.* (1993) detected a lower frequency of injury in garter snakes (*Thamnophis sirtalis* and *T. sauritus*) between 250–290 mm SVL relative to smaller neonates and larger adults. They suggested that injured juveniles do not survive their first year, possibly because of the stresses associated with hibernation. However, our data show no trend of especially low injury in small field-caught snakes and fitting a quadratic regression to the data in Fig. 2A did not reveal one either.

Diurnal activity, including basking behaviour, may make snakes particularly prone to being injured (Mushinsky & Miller, 1993). Thus, larger snakes might also be more likely to acquire injuries because they spend more time in the open than smaller snakes, for which such exposure is presumably more risky. Our finding that snakes found under cover are more likely to be small is consistent with this hypothesis and with Gregory's (1984) similar observations for three species of *Thamnophis*. Olson & Warner (2003) made similar findings for the colubrids, *Lampropeltis calligaster* and *Coluber constrictor*, but not for *Thamnophis sirtalis*. However, caution should be exercised in interpreting such data in terms of activity patterns. First, the frequency of snakes found under cover is only a correlate of relative activity in the open, which should be measured directly. This will be a challenge for small snakes, for which radiotelemetry is not possible. Second, if larger snakes require larger (or otherwise different) cover objects, then sampling only relatively small cover objects will necessarily bias a sample towards smaller snakes. Our study site lacks easily sampled natural cover objects, so we relied exclusively on artificial cover objects for finding hidden snakes. Although most of these cover objects appeared to be large enough for adult snakes, without testing specific requirements we cannot be sure. Presumably, natural cover, including the dense vegetation at the site, is important for snakes of all sizes, but cover objects, particularly those made of materials that retain heat, remove the risks inherent in basking, particularly for small snakes. For example, Mertens (1995) found that black plastic sheets were especially useful for catching smaller grass snakes. Alternatively, even if small and large snakes were to bask in the open equally often, larger

snakes might be more obvious to visual predators and hence more likely to attract the attention of those predators and be injured (Leavesley, 1987).

What causes injuries in grass snakes? An assumption in most studies, including this one, is that injuries are the result of encounters with predators, but this is generally not supported by direct observation, thus limiting the inferences that we can draw. At our study site, we have not witnessed any encounters between grass snakes and potential predators, which we assume are mainly birds and mammals. Injury due to intraspecific aggression is improbable; even male grass snakes competing for mates do not exhibit overt agonistic behaviour, such as biting, towards one another (Madsen & Shine, 1993b; Luiselli, 1996). However, we cannot rule out other possible causes of injury.

One possibility is wounding by dangerous prey, but grass snakes at Fordwich feed mainly on anurans (Gregory & Isaac, 2004), which lack defensive mechanisms that are likely to inflict injury on a predator. Grass snakes at Fordwich also eat mammals, which could cause injury, but the mammals eaten are mainly very small, including nestlings. Although snakes that eat nestling rodents might be subject to injury from mothers defending their young (Hoyer & Stewart, 2000b), mammal-eating in grass snakes at our study site is not restricted to the largest snakes (Gregory & Isaac, 2004) and thus seems an unlikely explanation of the higher frequency of injury in larger snakes.

Some minor scars could be attributable simply to wear-and-tear, such as abrasions from scraping against vegetation or hard substrates. Others might be due to infection or disease; for example, loss of the tail tip in *Elaphe subocularis* can result from parasitization by ticks (Degenhardt & Degenhardt, 1965). Finally, although we assumed in our analyses that hatchlings lack deformities that resemble injuries, we cannot entirely dismiss this possibility. Our sample of hatchlings was small and all eggs were incubated over a narrow range of temperatures (27–29°C). Townson (1990), again from a small sample, found that eggs incubated at higher temperatures yielded some snakes with deformed tails (although none of the deformed babies hatched), so that further experimentation is needed to obviate this possibility in surviving hatchlings. Live hatchlings with missing tails apparently do occur, but rarely (one observation; P. de Wijer, pers. comm.).

Presumably, injuries also are related in some way to defensive behaviour, either because animals exhibiting particular kinds of defensive behaviours are more likely to be injured (Seligmann *et al.*, 2003) or because animals that have been injured respond by changing their defensive behaviour (Willis *et al.*, 1993). One kind of injury that seems clearly linked to defensive behaviour is tail loss in the natricines, *Nerodia sipedon* (Fitch, 1999) and *Thamnophis sirtalis* (Cooper & Alfieri, 1993), as well as the colubrine, *Coluber constrictor* (Fitch, 1963).

In all three of these species, snakes that are grasped by the tail often will twist the body vigorously, apparently attempting to break the tail (sometimes succeeding in doing so; Gregory, pers. obs.) and then escaping. This autotomizing behaviour is more common, and presumably more effective, in larger individuals because they have sufficient body mass to apply the necessary force to break the tail (Fitch, 1999). If so, this is an additional reason why larger individuals in these species should have a higher incidence of injury than smaller snakes. Surprisingly, however, *Natrix natrix*, despite its relatively large size, its general ecological similarity to *Nerodia* and *Thamnophis*, and its close phylogenetic relatedness to them, only occasionally rotates the body when held by the tail (C. Reading, pers. comm.) and we have not seen such behaviour at our study site. Thus, perhaps not all instances of tail loss in *Nerodia* and *Thamnophis* are attributable to this particular defensive behaviour.

Like other studies of injury patterns in snakes, our work raises more questions than answers. It will be a challenge to extend work in this area because we lack the most fundamental natural history observations that are relevant to its study (e.g. direct observations of close encounters between snakes and their predators). Nonetheless, mark-recapture and other methods should at least allow us to begin comparing survivorship and reproductive success of injured and uninjured animals in species that are particularly amenable for field study. In short, research on injury rates in snakes needs to move beyond the descriptive stage and begin to test the consequences of injuries at the population level.

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GEOGRAPHIC VARIATION IN DIET COMPOSITION OF THE GRASS SNAKE (*NATRIX NATRIX*) ALONG THE MAINLAND AND AN ISLAND OF ITALY: THE EFFECTS OF HABITAT TYPE AND INTERFERENCE WITH POTENTIAL COMPETITORS

LUCA LUISELLI¹, ERNESTO FILIPPI¹ AND MASSIMO CAPULA²

¹Centre of Environmental Studies "Demetra", and F.I.Z.V. (Ecology), Rome, Italy

²Municipal Museum of Zoology, Rome, Italy

The diet of grass snakes (*Natrix natrix*) on the mainland and an island of Italy was compared by pooling literature data and original data. A total of 535 prey items were recorded (444 prey items from specimens >40 cm SVL), but the number of items was very variable between sites. Body lengths (both sexes) varied between geographical areas, and females were larger than males in all study areas. Specimens from the island (central Sardinia) and from one mainland mountainous locality (Duchessa Mountains) were significantly smaller than those from all the other localities. Amphibians were the main prey for both sexes, but females ate more toads and fewer frogs or tadpoles than males; females also consumed more rodents than males. There was a strong effect of locality on diet composition i.e. newts/salamanders were found only in two montane areas; hylids were found only in the single island area; and rodents were commonly preyed upon only at a single mainland locality. Two lizard corpses (*Podarcis muralis*) were scavenged by grass snakes at a mainland locality. The presence of the piscivorous snake *Natrix tessellata*, a potential competitor for food, did not have any apparent effect on the food types eaten by grass snakes because grass snakes consumed fish when sympatric with *N. tessellata*, but not at other sites. The dietary variation exhibited by grass snakes suggests that, by shifting their diets to other prey, they might be able to persist in areas where their usual natural prey has declined drastically, but this remains to be demonstrated.

Key words: body sizes, Colubridae, competition, food habits

INTRODUCTION

It is widely recognized that our understanding of the evolutionary ecology of snakes (e.g. habitat use, activity patterns, etc.) can be aided by detailed data on feeding ecology (e.g. Arnold, 1993; Cundall & Greene, 2000). Diet variation associated with differences in geography and habitat may be particularly useful in this respect (Gregory, 1984; Shine, 1987; Luiselli & Angelici, 2000; Henderson, 2002; Luiselli *et al.*, 2002; Luiselli, 2003). In addition, it has been recently demonstrated in some colubrids from tropical Africa (*Natriciteres* spp.) that the presence of a potential competitor for food (*N. variegata*) may produce a shift in the diet composition of the target species (*N. fuliginoides*). The result is that where species are sympatric, there is a partitioning of food resources between the two predator species and a reduction in the diversity of prey taken by the target species (Luiselli, 2003). The generality of this ecological phenomenon for snakes cannot be tested at present because, for most species, we do not have enough data to compare diets from areas where they live alone and areas where they coexist with potential competitors. However, food resource partitioning has been observed in several communities of snakes from both temperate and tropical regions (for a review, see Mushinsky, 1987), suggesting that the

mechanism of dietary shifts observed in Afrotropical *Natriciteres* (see Luiselli, 2003) may be relatively widespread.

Data on these issues are scarce. In Europe, geographic variation in diets has been studied only for *Coronella girondica* (Luiselli *et al.*, 2001), and microhabitat variation in diets has been studied only for *Elaphe longissima* (Capula & Luiselli, 2002; Gomille, 2002), *Elaphe quatuorlineata* (Filippi *et al.*, 2005) and *Vipera berus* (Luiselli & Anibaldi, 1991; Völkl & Thiesmeier, 2002). To date, the effects of potential competitors on diet composition of any European snake species has not been tested.

The diet of the grass snake (*Natrix natrix*) has been carefully studied in Italy over the last 15 years, but detailed data also are available for other conspecific populations from elsewhere (e.g. see Kabisch, 1974; Kratzer, 1974; Beschov & Dushkov, 1981; Madsen, 1983; Drobenkov, 1995; Reading & Davies, 1996; Gregory & Isaac, 2004). In this paper, we present detailed data on the diet of Italian grass snakes on one island and in several mainland habitats. We report on variations in grass snake diet that can be related to differences in geography, habitat and the effects of the presence of a potential competitor (*Natrix tessellata*).

In particular, we attempt to answer the following questions. Firstly, are there any significant differences in the diet composition of male and female grass snakes in areas with different climate and habitat con-

Correspondence: L. Luiselli, Centre of Environmental Studies "Demetra", and F.I.Z.V. (Ecology), via Olona 7, I-00198 Rome, Italy. *E-mail:* lucamlu@tin.it

ditions on the mainland and an island of Italy? The rationale behind this question is based on the fact that, in natricine snakes, females are usually bigger than the males (e.g., Madsen, 1983; Madsen & Shine, 1993a; Luiselli *et al.*, 1997; Luiselli, 2003; Shine, 2003; Shine *et al.*, 2003; Gregory & Isaac, 2004). Such body size differences may parallel differences in prey size and type. These parallels have been shown in other snake species, e.g. *Acrochordus arafurae* (Shine, 1986; Camilleri & Shine, 1990; Houston & Shine, 1993) and *Python regius* (Luiselli & Angelici, 1998), and we may therefore expect them to occur in *N. natrix*. Another reason for dietary differences between the sexes is the higher nutritional requirements of reproductive females compared to males (Bonnet *et al.*, 1998, 2001a; Gregory & Skebo, 1998; Shine, 2003); males often do not feed during the breeding season, whereas females are likely to select high-energy foods. Secondly, inter-population differences in diet may be related to weather/climate/habitat conditions, which in turn may affect energetic requirements (Capula & Luiselli, 2002). Secondly, do grass snakes exhibit shifts in feeding ecology associated with the presence or absence of *Natrix tessellata*, a closely related potential competitor which is also abundant and widespread in Italy (see Bruno & Maugeri, 1990; Luiselli & Rugiero, 1991; Filippi *et al.*, 1995)? This type of pattern may result in an apparent food partitioning between coexisting snakes, and has been demonstrated in another genus of natricine snakes, i.e. the small-sized *Natriciteres* species from tropical Africa (Luiselli, 2003).

We also address some general management implications that can be derived from the comparative data of the present study.

MATERIALS AND METHODS

The study is based on original field research conducted from March 1985 to May 2003 by us and our associates (primarily Drs U. Agrimi, C. Anibaldi, D. Capizzi, and L. Rugiero), with additional datasets from our own published research on *N. natrix* (e.g. Luiselli & Rugiero, 1991; Capula *et al.*, 1994; Luiselli *et al.*, 1997; Filippi & Luiselli, 2002).

STUDY AREAS

In total, six study areas (five on mainland Italy and one on the island of Sardinia), representing a range of different habitats and climatic conditions, are compared in this paper (Table 1). Although there is little genetic or morphological differentiation among mainland populations, Sardinian populations are extremely divergent and are classified as a different subspecies (*Natrix natrix cetti*; see Thorpe, 1975, 1979 for morphological evidence; Capula, unpublished data for genetic evidence).

METHODS

Field methods were nearly identical at all study areas, and are detailed in the original literature sources (see Table 1). Here, we summarize the main methodological points.

Fieldwork was conducted under all climatic conditions. We searched for grass snakes along standardized routes in the various microhabitats frequented by snakes at the study areas. We captured snakes by hand, but additional free-ranging specimens were captured by pitfall traps with drift fences. We always recorded the site of capture and the habitat at each capture site. Each snake

TABLE 1. List of study areas, including details of general habitats, presence or absence of the potential competitor (*Natrix tessellata*), and pertinent literature source.

Study area	Habitat features	Reference
MAINLAND ITALY		
La Marcigliana (Rome)	cultivated land; 50 m a.s.l.; Mediterranean climate; <i>Natrix tessellata</i> not present.	this paper
Palidoro (Rome)	artificial canal, 10 m a.s.l.; Mediterranean climate <i>Natrix tessellata</i> present.	Luiselli & Rugiero, 1991
Tolfa Mountains (Rome)	permanent stream; 350 m a.s.l.; Mediterranean climate <i>Natrix tessellata</i> present.	Filippi <i>et al.</i> , 1996
Duchessa Lake (Apennines)	glacial lake; 1800 m a.s.l. cold climate <i>Natrix tessellata</i> not present.	Filippi & Luiselli, 2002
Sella Nevea (Alps)	detrital cone along a Swiss pinewood; 1100 m a.s.l.; alpine climate <i>Natrix tessellata</i> not present.	Luiselli <i>et al.</i> , 1997
ISLAND AREA		
Oliena (Nuoro, Sardinia)	Mountain stream; 800 m a.s.l.; cool Mediterranean climate <i>Natrix tessellata</i> not present.	Capula <i>et al.</i> , 1994

was sexed, measured for snout-vent length (SVL, to the nearest ± 1.0 mm), weighed with an electronic balance, and individually marked by ventral scale clipping for future identification. Then, the snakes were palpated in the abdomen until regurgitation of ingested food or defaecation occurred. In addition, specimens found already dead during our surveys (e.g. snakes killed by farmers, or by cars, etc.) were dissected to determine if prey was present. We identified prey items to the lowest taxonomic level possible. We estimated the mass of prey items at the time of ingestion, when possible, by comparing the item to intact conspecifics of various sizes from our own personal collection, or measuring the fresh biomass in perfectly preserved items. We utilized data collected from both stomachs and faeces. Generally, each specimen contributed data from either only stomach contents or faeces but not both (i.e. to avoid dual counts of the same food item), unless faecal and stomach samples contained obviously different material (e.g. mammal hair vs. an amphibian), in which case both sources were used in the data set. In the case of stomach contents, we always counted number of prey items rather than number of snakes with a particular prey type.

Vouchers (of both prey and predators) were deposited in the herpetological collections of the Centre of Environmental Studies 'Demetra' (Rome), F.I.Z.V. (Rome), and Municipal Museum of Zoology (Rome).

All statistical tests were done with alpha set at 0.05. Means are followed by ± 1 SD. For all analyses we separated small specimens (<40cm SVL) from large specimens based on two criteria: (1) they were certainly immature; (2) due to the fact that juveniles are more elusive than adults, our samples of small snakes were strongly biased, i.e. they varied considerably from study area to study area, and did not allow for robust statistical comparisons at some areas.

TABLE 2. Composition of the diet of grass snakes in a cultivated area in Mediterranean central Italy (Marcigliana, Rome). These data are based on examination of 71 adult males and 54 adult females, out of which 33 males and 20 females contained identifiable prey in either their stomach or faeces. Numbers represent the number of individual prey, not the number of snakes containing a given prey type.

Prey types	No. in males	No. in females
AMPHIBIANS		
<i>Bufo bufo</i>	8	6
<i>Bufo viridis</i>	2	3
<i>Rana bergeri</i>	6	1
MAMMALS		
<i>Microtus savii</i>	10	11
<i>Rattus rattus</i>	3	-
<i>Mus domesticus</i>	1	-
<i>Crocidura</i> sp.	4	-

RESULTS

DIET OF GRASS SNAKES IN AN AGRICULTURAL AREA

The only original data set presented in this paper concerns a grass snake population from an agricultural area in Mediterranean central Italy (La Marcigliana, see Table 1). The composition of the diet of this grass snake population is summarized in Table 2. These data are based on an examination of 71 adult males and 54 adult females, out of which 33 males (46.5%) and 20 females (37%) contained identifiable prey in either their stomachs or faeces. A total of 34 prey items were collected from males, and 21 prey items from females. Small mammals were eaten slightly more often than amphibians in both males (53% versus 47% of the dietary spectrum) and females (52.4% versus 47.6% of the dietary spectrum). In both sexes, the most common prey items were Savi's voles, *Microtus savii*, (accounting for 29.4% of the prey items found in males, and 52.4% of those found in females) and common toads *Bufo bufo* (23.5% of prey items in males, and 28.6% in females). Savi's voles were by far the commonest rodents in the agricultural lands of the study area (Capizzi & Luiselli, 1996a,b), whereas common toads were the most abundant amphibians in the same habitat (Capizzi & Luiselli, unpublished).

COMPARING THE STUDY AREAS

Mean body sizes. Mean SVL of adult (i.e. >40 cm) grass snakes varied between sites (Table 3). Females were significantly longer than males at all study areas (one-way ANOVA, significance is always at least $P < 0.001$). Male SVL varied significantly between geographical regions (one-way ANOVA, $F_{5,207} = 38.6$, $P < 0.01$), and a Tukey HSD post-hoc test demonstrated that specimens from the island locality (Oliena) and from one mainland mountainous locality (Duchessa Mountains) were not significantly different from each other, but were significantly smaller than those from all the other localities, whose male body sizes were similar. An identical pattern was found for females (overall one-way ANOVA, $F_{5,141} = 44.3$, $P < 0.01$).

TABLE 3. Mean length (SVL) \pm SD for grass snakes at all the study areas. Detailed data are presented in the original literature sources (see the text). In bold are the populations that differed significantly in mean body length from all other populations, but not from each other (for statistical details, see the text).

Study area	Body length (cm) males	Body length (cm) females
La Marcigliana	71.9 \pm 10.4 (n=71)	87.5 \pm 3.6 (n=54)
Palidoro	69.3 \pm 15.6 (n=57)	84.8 \pm 14.3 (n=39)
Tolfa Mountains	72.3 \pm 12.8 (n=21)	89.7 \pm 18.2 (n=19)
Duchessa Lake	51.4\pm17.6 (n=8)	62.2\pm28.9 (n=4)
Oliena	47.3\pm4.7 (n=10)	62.7\pm11.6 (n=8)
Sella Nevea	71.3 \pm 10.2 (n=43)	82.1 \pm 13.7 (n=20)

TABLE 4. Summary of diet data of grass snakes from the various study areas. Data are pooled from all localities, and only grass snake specimens longer than 40 cm SVL are considered.

Prey types	No. in females	% in females	No. in males	% in males
FISHES				
Gobiidae	1	0.4	3	1.4
Cyprinidae (unidentified)	4	1.8	3	1.4
<i>Anguilla anguilla</i>	1	0.4	3	1.4
AMPHIBIANS				
<i>Bufo</i> adults	41	18.3	12	5.4
<i>Bufo</i> metamorphs	51	22.8	57	25.9
<i>Bufo</i> tadpoles	23	10.3	36	16.4
<i>Rana</i> adults	43	19.2	59	26.8
<i>Rana</i> metamorphs	4	1.8	9	4.1
<i>Rana</i> tadpoles	6	2.7	2	0.9
<i>Hyla</i> adults	3	1.3	3	1.4
<i>Hyla</i> tadpoles	2	0.9	4	1.8
Newts/salamanders	15	6.6	14	6.4
Amphibia (unidentified)	0	0	1	0.4
REPTILES				
Lizards	3	1.3	2	0.9
BIRDS				
Passeriformes	2	0.9	0	0
MAMMALS				
Rodents	25	11.2	12	5.4
TOTAL	224	100.0	220	100.0

Diets. Detailed data on the diet composition of the various grass snake populations compared here are presented in the original bibliographic sources, but a list of the various prey items is reported in Appendix 1. When we pooled all the studied populations, there was a total of 535 recorded prey items, although the number of items was very variable between sites due to different lengths of study period, and differences in the abundance of grass snakes between sites.

When we excluded small specimens (those <40 cm SVL) for which we had good data from only a single locality (i.e. Palidoro; see Luiselli & Rugiero, 1991, where they fed mainly on anuran tadpoles), we were left with a total of 224 prey items from adult females and

220 prey items from adult males (Table 4). Amphibian prey accounted for the greatest part of the diet in both females (83.9 % of the total number of prey items) and males (89.5 %). The sexes did not differ significantly in terms of frequency of consumption of amphibians (contingency table χ^2 , with comparisons of each prey type, one at a time, against all others: $\chi^2=1.61$, $df=1$, $P=0.20$), fish prey ($\chi^2=3.12$, $df=1$, $P=0.08$), reptile prey ($\chi^2=0.01$, $df=1$, $P=0.93$), or bird prey ($\chi^2=1.98$, $df=1$, $P=0.16$). However, the sexes did differ significantly in terms of frequency of consumption of mammals ($\chi^2=20.21$, $df=1$, $P<0.001$), with males taking fewer rodents than females. In addition, when we looked in more detail at the various types of amphibians consumed some significant intersexual differences emerged i.e. females took more adult *Bufo* than males ($\chi^2=88.86$, $df=1$, $P<0.001$), but fewer *Bufo* tadpoles ($\chi^2=12.99$, $df=1$, $P<0.001$) and adult *Rana* ($\chi^2=11.48$, $df=1$, $P<0.001$) than males.

In addition to differences between the sexes, there was a strong effect of locality on grass snake diet as (1) newts/salamanders were found only in two mountain areas (Duchessa Lake and Sella Nevea); (2) treefrogs were found only in a single area (Oliena); and (3) rodents were commonly preyed upon only at a single locality (La Marcigliana).

Amphibian prey were dominant at almost every study area apart from La Marcigliana (Fig. 1), and the percentage of amphibians in the diet was not significantly

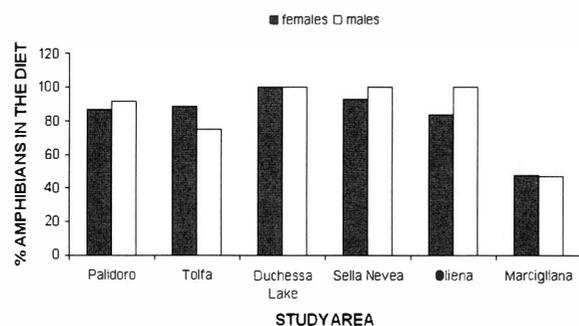


FIG. 1. Variation between sites in the percentages of amphibians in the diet of grass snakes of both sexes. For the total number of prey items at each study area, see Appendix 1.

related to the altitude of the various study areas in either females ($r^2=0.42$, regression: $F_{1,4}=2.94$, $P=0.16$) or males ($r^2=0.42$, regression: $F_{1,4}=2.91$, $P=0.16$). The two regression lines did not differ significantly in terms of slope (heterogeneity of slopes test: $F_{1,8}=0.03$, $P=0.87$) or y-intercepts (heterogeneity of intercepts: $F_{1,8}=0.09$, $P=0.62$).

Feeding on carrion was documented in two cases, where one adult male and one adult female from the Tolfa Mountains site ingested lizard corpses (*Podarcis muralis*). Evidence of carrion-feeding by grass snakes in these cases was proved by (1) the direct observation of a snake collecting and ingesting a lizard squashed on the road, and (2) by a lizard head, in perfect conditions (so clearly ingested within a short time), but without its body, regurgitated by another grass snake. In this latter case, it is likely that this lizard was killed by a bird of prey, which ingested the body of the lizard after having removed the head (which was subsequently ingested by the grass snake).

MONTHLY VARIATION IN DIETS

For reasons of climatic homogeneity, we considered the monthly variation in prey consumption by grass snakes from three areas of Mediterranean central Italy, i.e. Palidoro, Tolfa Mountains; and La Marcigliana (Table 5). The data collected from some other localities (Duchessa Lake, Oliena) were too few to be analysed in terms of monthly intervals, and data from Sella Nevea cannot be compared directly because the climate of this region is much harsher than that of the above areas. For both sexes, more prey were consumed during the spring months, particularly in May (Fig. 2). This seasonal pattern is partly explained by the presence of multiple prey items of a small size (e.g. anuran tadpoles) in the guts of some spring-captured grass snakes, but it also perhaps reflects a need for intense foraging after the hibernation period in this species. Indeed, if we consider the proportion of fed specimens (after having pooled data into two-month intervals in order to reach an adequate sam-

TABLE 5. Summary of the monthly distribution of diet data of grass snakes at three study areas in Mediterranean central Italy. Numbers of prey items are indicated in italics for females and boldface for males.

Prey types	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov
PALIDORO									
<i>Anguilla anguilla</i>			1	<i>1,1</i>					
<i>Cyprinidae</i> sp.			<i>1</i>	2	<i>2</i>				
<i>Rana bergeri</i>			<i>1,2</i>	2,1	<i>1,2</i>	<i>1</i>			
<i>Bufo bufo</i> tadpoles	19,28	<i>4,7</i>							
<i>Bufo bufo</i> metamorphs		6,3	12,10						
Amphibia (unidentified)					1				
<i>Chalcides chalcides</i>			1						
<i>Rattus</i> sp.								<i>1</i>	
<i>Turdus merula</i>	<i>1</i>								
<i>Passer</i> sp.		<i>1</i>							
TOLFA MOUNTAINS									
<i>Anguilla anguilla</i>				1					
<i>Cyprinidae</i>				<i>1</i>					
<i>Gobiidae</i>				<i>1,1</i>	<i>1,1</i>	1	1		
<i>Bufo bufo</i> adults		<i>1</i>	<i>4</i>	<i>3</i>	<i>1</i>		<i>1</i>		
<i>Bufo bufo</i> metamorphs		9,6	1						
<i>Rana italica</i>				1	1	1		<i>1</i>	
<i>Rana bergeri</i>		<i>1,1</i>	<i>3,5</i>	<i>1,2</i>	1				
<i>Rana bergeri</i> tadpoles			<i>1</i>	4,1	<i>1,1</i>				
<i>Podarcis muralis</i>		<i>1</i>						1	
<i>Apodemus sylvaticus</i>							1		
MARCIGLIANA									
<i>Bufo bufo</i>		<i>1,</i>	<i>2,4</i>	<i>1,3</i>			<i>1,</i>	<i>1,1</i>	
<i>Bufo viridis</i>		3,1	1						
<i>Rana bergeri</i>			<i>1,3</i>	1	1	1			
<i>Microtus savii</i>	<i>1</i>	<i>2,1</i>	<i>4,1</i>	2				<i>2,5</i>	<i>1,1 1</i>
<i>Rattus rattus</i>		1						2	
<i>Mus domesticus</i>								1	
<i>Crocidura</i> sp.	1		1					1	1

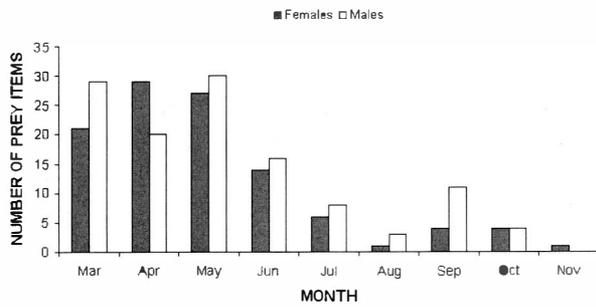


FIG. 2. Monthly numbers of prey items found in adult grass snakes of both sexes after pooling data from three localities of Mediterranean central Italy (Palidoro, Tolfa Mountains, and Marcigliana) that were carefully surveyed throughout the various periods of the year. Note the large number of prey items found during the spring months.

ple size for statistical comparisons), it also resulted in a peak of specimens with food in the stomach in the spring months (Fig. 3; March-April and May-June being the major peaks; at least $P < 0.05$; χ^2 test comparisons with all other two-month-intervals; differences not significant between the two above-mentioned intervals). Moreover, the feeding peak in spring (Figs. 2 and 3) mirrors a peak in above-ground grass snake activity that is seen in the same months at the Tolfa Mountains' site (Fig. 4; and see Filippi, 1995, page 102).

POTENTIAL COMPETITORS AND GRASS SNAKE DIET

Fish prey were found in only two out of six populations of grass snakes, and in both cases *Natrix tessellata*, a potential competitor feeding on fish (see Luiselli & Rugiero, 1991), was present at the site. Surprisingly, grass snakes did not eat fish in the four areas where *N. tessellata* was absent, although fish were present at one of these sites (see Discussion). This result was not an artifact of the sample sizes, as there were no significant relationships between the total number of prey items taken by grass snakes in each area and the

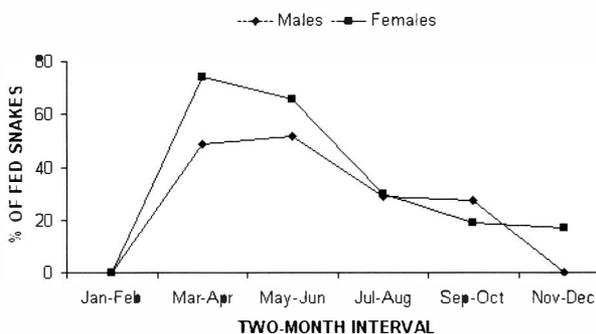


FIG. 3. Variation in the percentage of grass snakes with prey in the stomach in relation to period of capture. Data are included for three Mediterranean localities (La Marcigliana, Palidoro, and Tolfa Mountains). Data were grouped into two-month-intervals in order to reach an adequate sample size for statistical comparisons. Sample sizes: Jan-Feb, No. males=10, No. females=8; Mar-Apr, No. males=41, No. females=23; May-Jun, No. males=31, No. females=32; Jul-Aug, No. males=31, No. females=27; Sep-Oct, No. males=33, No. females=16; Nov-Dec, No. males=3, No. females=6.

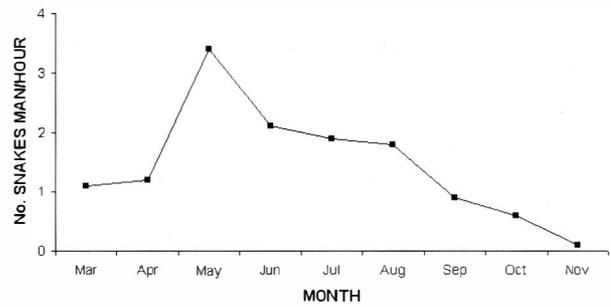


FIG. 4. Monthly patterns of above-ground activity of grass snakes at Tolfa Mountains evaluated in terms of mean number of specimens observed active per three hours of field effort made by two researchers working independently (for more details, see Filippi, 1995). Male and female data are combined.

number of fish eaten ($r=0.52$, regression: $F_{1,4}=1.50$, $P=0.29$). Also, the proportion of amphibians eaten was not much different between areas with or without *N. tessellata* (see Fig. 1).

DISCUSSION

COMPARING THE VARIOUS STUDY AREAS

Although it is generally accepted that grass snakes frequently feed upon anuran amphibians (e.g. see Kabisch, 1974; Arnold & Burton, 1978; Beshkov & Dushkov, 1981; Bruno & Maugeri, 1990) no previous study has examined local variation in the diet of the grass snake over a well defined geographical region, such as Italy. Thus, this study may be useful for discovering patterns of diet variation that have been masked by studies conducted at single sites. Furthermore, the information gained may be important from a conservation perspective.

Our study shows that the dietary variation of grass snakes is high. This is demonstrated by the preponderance of alternative prey to anurans in areas with particular habitat and climate characteristics. For instance, newts and salamanders are the main prey in high mountain regions with a cold climate, and voles are the main prey in an agricultural zone without pristine habitat patches. We interpret these dietary differences to be a consequence of differences in prey abundance (and perhaps relative availability of prey of different sizes) because all the studied mainland populations are genetically very close (M. Capula, unpublished data) and there is thus probably little influence of phylogeny on dietary variation. The smaller body sizes of grass snakes from Duchessa mountains in comparison to the other mainland localities (see Table 3) may be a consequence of limited snake activity in a very cold climate (i.e. short feeding period). If this is the case, it is another instance of the effects of plasticity in body sizes and life history traits of snakes (Madsen & Shine, 1993b; Forsman, 1996; Forsman & Shine, 1997; Queral-Regil & King, 1998; Bonnet *et al.*, 2001b). In this regard, it is noteworthy that in Swedish grass snakes, both the growth rates and the asymptotic body sizes were reduced because of low food availability, without any genetic modifications

of the populations (Madsen & Shine, 1993b). By contrast, the small body size of Sardinian grass snakes is more likely to be genetic, as (1) the average body lengths of the individuals of the subspecies *cetti* are known to be smaller than those of mainland Italy (Thorpe, 1975, 1979; Stefani, 1983), and (2) other insular snake populations offer some dramatic examples of geographic variation in body size (e.g. Shine, 1987; Schwaner & Sarre, 1988; King, 1989).

The effects of phylogenetic distance on food variations may, however, be relevant in the case of the genetically isolated and diverging populations from Sardinia (M. Capula, unpublished data), which showed a dietary spectrum remarkably different from that of their mainland conspecifics. Their diet consists only of hylids and lizards, which are rarely eaten on the mainland, and this may also be related to (1) the smaller size of Sardinian grass snakes compared to mainland populations (Stefani, 1983; Capula *et al.*, 1994); (2) the different prey base available in Sardinia compared with mainland Italy; and (3) the drier climate of the Sardinian mountains compared to the Apennines and the Alps.

MONTHLY VARIATION IN DIETS

Our data indicate that there is a higher probability of finding food in the stomachs of grass snakes captured in spring than in summer or autumn, at least in three Mediterranean study areas (Table 5). These results are in agreement with data on British grass snakes (Gregory & Isaac, 2004), which also showed a higher probability of having food in the stomachs during spring than in summer. However, British grass snakes also had a high probability of having food in stomachs during autumn (Gregory & Isaac, 2004), whereas the same pattern was not found in Italian conspecifics. We suggest that these differences are strongly linked to the different climates and, consequently, the likelihood of snakes finding amphibians active in the open. Indeed, in Mediterranean areas the weather in early autumn (September) is still very hot and dry, many ponds and streams are desiccated, and it is very hard to find frogs and toads active in the open. Hence, there is little reason for grass snakes to move around in search of prey. Conversely, the British weather is much wetter, and it is likely that the same problems in finding amphibians do not apply to British grass snakes. In European *Vipera* species, it has also been demonstrated that feeding rates tend to be lower in summer than in spring or autumn in the dry Mediterranean climate (Luiselli & Agrimi, 1991), whereas they are higher in summer than in spring or autumn in cooler and wetter regions (Brito, 2004). Thus, it seems evident that the foraging periods of European snakes are strongly related to the proximate climatic conditions of the various study areas.

POTENTIAL COMPETITORS AND GRASS SNAKE DIET

Interactions with a potential food competitor, i.e. the piscivorous *N. tessellata*, did not have any of the expected effects on the food types consumed by grass

snakes because grass snakes also consumed fish, which were generally not consumed in the absence of *N. tessellata*. We believe that grass snakes fed on fish in the two areas where *N. tessellata* is present merely because fish are an abundant food resource in these areas, and high dietary overlap can therefore be tolerated between the two snake species. Fish were also abundant at Duchessa Lake, but this is a high mountain site with cold water year-round and so it is likely that grass snakes cannot spend a long time foraging aquatically, and consequently cannot prey efficiently on fast-swimming animals such as fish. The snakes at Duchessa Lake are therefore possibly forced to feed upon newts, which are much easier to capture in cold water because they are slower than fish of a comparable size. Additionally, newts are found in shallower water (< 30 cm depth) than fish (generally > 90 cm depth), i.e. in sites where the water temperature is higher (E. Filippi & L. Luiselli, unpublished data).

The dynamics of coexistence of *N. natrix* and *N. tessellata* apparently differ from those of the Afrotropical marsh colubrids *Natriciteres variegata* and *Natriciteres fuliginoides* (see Luiselli, 2003), although in both cases a pattern of food resource partitioning seems to be present. The mechanism of food resource partitioning in *Natriciteres* species appears to be induced by the competition between two species which, when in separate locations, have generalist dietary preferences. In our study sites where the two *Natrix* species coexist, however, diet partitioning between the species seems to occur as *N. tessellata* seems to specialise on fish, and *N. natrix* on amphibians. Thus, in this latter case, it seems very unlikely that present day competition can explain the coexistence of these two species. Moreover, whereas the two *Natriciteres* species are nearly identical in terms of both body size and habitat preferences (Luiselli, 2003), *N. tessellata* is considerably smaller and more aquatic than its congener (Bruno & Maugeri, 1990). Perhaps *N. tessellata* has undergone a previous evolutionary character displacement for specialized feeding on fish. By contrast, *N. natrix* is not a specialist, but simply an adaptable species that usually prefers anurans, but feed on fish (or other prey types) if conditions allow. Concerning the island grass snake population, its potential competitor was not *N. tessellata* (which is absent from Sardinia, see Bruno & Maugeri, 1990), but the viperine snake *Natrix maura*, which is widespread and abundant in Sardinia (Bruno & Maugeri, 1990), and has been suspected to be a major competitor for *N. natrix cetti* (Stefani, 1983). Indeed, *N. maura* also has a dietary spectrum similar to that of *N. natrix*, with anuran amphibians and fish being the main prey (Santos & Llorente, 1998), and in Sardinia its main prey species were *Hyla sarda* and *Discoglossus sardus* (Rugiero *et al.*, 2000). Although it is possible that the two species may indeed compete for food at our island study area given their similar dietary spectrums, the absence of other control areas in Sardinia prevents us from stressing any conclusion on this issue. At the moment we

are still collecting data on the coexistence of these two species at several study areas in Sardinia, and perhaps we will present the data in a forthcoming article.

CONCLUSIONS

Our review shows that grass snakes consume a wide variety of vertebrate prey. The grass snake also can be added to the list of species that will occasionally eat carrion (for a review, see DeVault & Krochmal, 2002). In addition, we have shown that grass snake populations differ in their dietary spectrums. As further evidence of the dietary variability of these snakes, we note that populations in Kent (southern England) are known to feed intensively on *Rana ridibunda*, which is an introduced species to Great Britain (Gregory & Isaac, 2004). Such data may have implications for the management strategies used in grass snake conservation, especially given that their main prey (amphibians) are declining globally (see Gardner, 2001, for a review), which suggests that grass snakes may become threatened in areas with vulnerable amphibian populations. However, the variable diet composition exhibited by grass snakes suggests that these snakes possibly can be preserved in areas where their usual prey has declined drastically, because they presumably can switch to alternative prey. How – and how fast – dietary shifts occur remains to be seen because evidence for such shifts so far is based only on differences between populations (this study, and see also Gregory & Isaac, 2004), not on observed changes within populations. Indeed, it is possible, but still unverified, that grass snakes may be plastic in diet, i.e. that the individuals change diet in response to changing conditions (Ford & Seigel, 1994), but it is also possible that the observed variation in diet arises from local adaptation and is actually non-plastic. By contrast, the decline of amphibians (mainly due to the introduction of non-native trout) has apparently caused a decline of the amphibian-eating natricine snake *Thamnophis elegans elegans* in the Sierra Nevada (Matthews *et al.*, 2002), and so it is more than likely that the responses of the various species of natricine snakes to changes in amphibian prey availability may vary substantially.

In conclusion, *N. natrix* is not a specialist on anurans (e.g. see Bruno & Maugeri, 1990) but an adaptable generalist with a preference for amphibians (e.g. Gregory & Isaac, 2004), and this information could be important for conservation if diet change can occur rapidly within a population.

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APPENDIX I

List of prey items of grass snakes at the various study areas. Appropriate references are included. All data are classified by sex, although in some cases it was not explicitly done in the original source.

PALIDORO (data from Luiselli & Rugiero, 1991): total number of prey items: 205; juveniles (shorter than 40 cm SVL) – *Anguilla anguilla* (n=1), Cyprinidae indetermined (n=1), *Rana bergeri* (n=1), *Bufo bufo* tadpoles (n=74), *Bufo bufo* metamorphs (n=16); females – *Anguilla anguilla* (n=1), Cyprinidae indetermined (n=3), *Rana bergeri* (n=4), *Bufo bufo* tadpoles (n=23), *Bufo bufo* metamorphs (n=18), *Rattus* sp. (n=1), *Turdus merula* (n=1), *Passer* sp. (n=1); males – *Anguilla anguilla* (n=2), Cyprinidae indetermined (n=2), *Rana bergeri* (n=5), *Bufo bufo* tadpoles (n=35), *Bufo bufo* metamorphs (n=13), Amphibia unidentified (n=1), *Chalcides chalcides* (n=1).

TOLFA MOUNTAINS (data from Filippi *et al.*, 1996): total number of prey items: 63; females – Cyprinidae unidentified (n=1), Gobiidae unidentified (n=2), *Bufo bufo* adults (n=10), *Bufo bufo* metamorphs (n=9), *Rana italica* (n=1), *Rana bergeri* (n=5), *Rana bergeri* tadpoles (n=6), *Podarcis muralis* (n=1); males – Cyprinidae indetermined (n=1), Gobiidae unidentified (n=3), *Anguilla anguilla* (n=1), *Bufo bufo* metamorphs (n=7), *Rana italica* (n=3), *Rana bergeri* (n=9), *Rana bergeri* tadpoles (n=2), *Podarcis muralis* (n=1), *Apodemus sylvaticus* (n=1).

DUCHESSA LAKE (data from Filippi & Luiselli, 2002): total number of prey items: 9; females – *Triturus carnifex* (n=2); males – *Bufo bufo* tadpoles (n=1), *Triturus carnifex* (n=6).

SELLA NEVEA (data from Luiselli *et al.*, 1997): total number of prey items: 190; females – *Rana temporaria* adults (n=26), *Rana temporaria* metamorphs (n=4), *Bufo bufo* adults (n=22), *Bufo bufo* metamorphs (n=26), *Salamandra atra* (n=6), *Triturus alpestris* (n=7), *Lacerta vivipara* (n=1), *Apodemus sylvaticus* (n=6); males – *Rana temporaria* adults (n=6), *Rana temporaria* metamorphs (n=9), *Bufo bufo* adults (n=2), *Bufo bufo* metamorphs (n=37), *Salamandra atra* (n=5), *Triturus alpestris* (n=3).

OLIENA (data from Capula *et al.*, 1994): total number of prey items: 13; females – *Hyla sarda* adults (n=3), *Hyla sarda* tadpoles (n=2), *Podarcis tiliguerta* (n=1); males – *Hyla sarda* adults (n=3), *Hyla sarda* tadpoles (n=4).

TAXONOMIC CHAOS IN ASIAN RANID FROGS: AN INITIAL PHYLOGENETIC RESOLUTION

LIQIAO CHEN¹, ROBERT W. MURPHY², AMY LATHROP², ANDRE NGO², NIKOLAI L. ORLOV³, CUC THU HO⁴ AND ILDIKO L. M. SOMORJAI⁵

¹*Department of Biology, East China Normal University, Shanghai, China*

²*Centre for Biodiversity and Conservation Biology, Royal Ontario Museum, Ontario, Canada*

³*Zoological Institute, Russian Academy of Sciences, Petersburg, Russia*

⁴*Institute of Ecology and Biological Resources, Nghai Do, Tu Liem, Hanoi, Vietnam*

⁵*Genetics Department, University of Cambridge, England, UK*

The taxonomy of ranid frogs is in a state of chaos, and Asian ranids are no exception. We undertook an investigation of the phylogenetic relationships of most major groups of Asian ranids using mitochondrial DNA sequences from the 12S, tRNA^{Val} and 16S genes. The resulting phylogenetic hypothesis had varying correspondence with the current taxonomy of the frogs at the subfamilial and generic levels. In order to maintain a taxonomy that reflects phylogenetic history, a number of taxonomic changes are proposed. Within subfamily Raninae, we recognize the genera *Rana*, *Amolops*, *Hylarana*, *Odorrana* and *Nidirana*. Recognition of *Huia* is not supported by our data and the recognition of *Pseudorana* is equivocal. Tribe Limnionectini is elevated to subfamily Limnionectinae and it contains *Limnionectes*, *Hoplobatrachus* and *Nanorana*. Membership in Genus *Limnionectes* is redefined. Recognition of genera *Paa* and *Chaparana* results in a paraphyletic taxonomy.

Key words: Anura, Asia, mtDNA, phylogeny, Ranidae

INTRODUCTION

For many years following the major revisions of Boulenger (1882, 1918, 1920), the taxonomy of ranid frogs was stable. Now it is in a state of chaos. Numerous generic and subgeneric shifts have been proposed, usually without an examination of phylogenetic relationships. Dubois (1986 (1987)) recognized six tribes within the Raninae (=Ranidae by most authorities). Among ranids, his Ranini included the genera *Altirana*, *Amolops*, *Batrachylodes*, *Micrixalus*, *Nanorana*, *Staurois*, and *Rana*, with the subgenera *Amietia*, *Hylarana*, *Paa*, and *Strongylopus*, and *Rana*. Dubois' Tomopternini only included the genus *Tomopterna*. The tribe Ptychadenini had *Ptychadena* and *Hildebrandtia*. His Dicroglossini contained *Ceratobatrachus*, *Conraua*, *Discodeles*, *Limnionectes* (with five subgenera: *Limnionectes*, *Bourretia*, *Fejervarya*, *Hoplobatrachus*, and *Taylorana*), *Occidozyga* (with two subgenera: *Occidozyga* and *Euphlyctis*), *Palmatorappia*, *Phrynoglossus*, *Platymantis*, and *Ingerana* (with *Ingerana* and *Liurana* as subgenera). The fifth tribe, Pyxicephalini, included *Pyxicephalus*. Finally, tribe *Ranixalini* had *Ranixalus*, *Nannophrys*, and *Nyctibatrachus*.

Higher taxonomy has continued to change. Dubois (1992) raised the tribe Dicroglossini to the subfamily level, Dicroglossinae. He recognized four tribes in this

subfamily: (1) Ceratobatrachini (*Ceratobatrachus*, *Discodeles*, *Ingerana*, *Palmatorappia*, *Platymantis*, and *Taylorana*), (2) Conrauini (*Conraua*), (3) Dicroglossini (*Euphlyctis*, *Occidozyga*, and *Phrynoglossus*), and (4) *Limnionectini* (*Hoplobatrachus* and *Limnionectes*). These arrangements have been controversial. Inger (1996) noted that tribe Limnionectini was demonstrably paraphyletic with respect to the Ceratobatrachini, Conrauini, and Dicroglossini. No evidence supported the monophyly of Limnionectini. It may be paraphyletic with respect to the Mantellinae and through the mantellines to the Rhacophoridae. Laurent (1951, 1979) and Ford (1993) questioned the monophyly of the Ranidae (*sensu stricto*) with respect to the family Rhacophoridae.

Recently, Chinese authorities have proposed numerous other changes, in particular generic reallocations. Fei *et al.* (1990 [1991]) described or erected a number of new generic combinations for many Chinese species. Further generic changes were made by Ye *et al.* (1993) and Fei (1999). These changes were made in the absence of a phylogenetic evaluation. Thus, we undertook an investigation of ranid relationships, particularly for representative south-east Asian genera and species. When we initiated this study, no phylogenetic evaluation of the group had been attempted at a higher taxonomic level, although one distance-based evaluation had been made (Wallace *et al.*, 1973). Subsequently, four other phylogenetic studies have reported on the relationships of ranid frogs, as discussed below.

Correspondence: R. W. Murphy, Centre for Biodiversity and Conservation Biology, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario, Canada M5S 2C6. E-mail: drbob@zoo.utoronto.ca

TABLE 1. Primers used for amplifying and sequencing fragments of RNA genes in the subfamily Raninae. Sequence position indicates the starting position of the primer in the *Xenopus laevis* genome and is preceded by the amplification direction as indicated by (H) heavy or (L) light strand.

Name	Sequence 5' to 3'	Sequence position	Reference
12S1L	CAAACCTGGGATTAGATACCCCACTAT	L2484	Kocher <i>et al.</i> (1989)
12S2H	AGGGTGACGGGCGGTGTGT	H2897	Kocher <i>et al.</i> (1989)
12S2L	ACACACCGCCCGTCACCCTC	L2917	Fu (1999)
16S3H	GTAGCTCACTTGATTTCCGGG	H3341	Fu (pers. comm.)
16S3L	CCCGAAATCAAGTGAGCTAC	L3362	Fu (pers. comm.)
16S1H	GGCTATGTTTTTGGTAAACAG	H3958	Modified from Palumbi (1996)
16S5H	CTACCTTTGCACGGTTAGGATACCGCGGC	H4040	Fu (2000)
16S1M	CCGACTGTTTACCAAAAACAT	L3955	Fu (1998)
16S2H	CCGGATCCCCGGCCGGTCTGAACTCAGATCAG	H4552	Palumbi (1996)

MATERIALS AND METHODS

SPECIMENS EXAMINED

Forty-five individuals, most of them south-east Asian ranines, were sequenced for three mitochondrial DNA genes. Additional sequence data from GenBank were used for the following species: *Rana pipiens* (X86247, X86318), *R. catesbeiana* (M57572), *R. temporaria* (Y11977), and *Xenopus laevis* (M10217). We used *X. laevis* as our initial outgroup taxon, and included an Asian treefrog (family Rhacophoridae, subfamily Rhacophorinae), *Polypedates megacephalus* (AF026350, AF026367), and an African mantelline (family Rhacophoridae, subfamily Mantellinae), *Laliostoma labrosum* (AF026354, AF026374), in our study to evaluate the monophyly of the family Ranidae as questioned by Laurent (1951, 1979) and Ford (1993). GenBank accession numbers, collection locality and voucher data for specimens sequenced in this study are given in an electronic Appendix downloadable from the Journal's website (<http://biology.bangor.ac.uk/~bss166/HJ/>). These tissue samples and most voucher specimens are preserved in the Royal Ontario Museum (ROM), or in the tissue collections of Jinzhong Fu (JF) and James P. Bogart (JPB) (Department of Zoology, University of Guelph). We also incorporated sequences from an analysis of fanged ranids (Emerson & Ward, 1998). These species include *Limnonectes acanthi* (U66120-21), several populations of *L. blythii* (U55262-3, U55269-70, U66114-15, U66126-27, U66130-31, U66134-37), *L. grunniens* (U66124-25), *L. ibanorum* (U66122-23), *L. ingeri* (U55268, U55275), *L. limnocharis* (U55265, U55272), *L. macrocephala* (66116-17), *L. macrodon* (U66132-33), *L. magna* (U66118-19), *L. paramacrodon* (U55267, U55274), *Limnonectes* sp. ("duboisii," a nomen nudum; Dubois, 1999) (U66112-13), and *Occidozyga laevis* (U66138-39). Taxonomic assignment of examined species generally follows Frost (2004).

DNA AMPLIFICATION AND SEQUENCING

Three ribosomal RNA genes, 12S, 16S, and tRNA^{Val} from the mitochondrial genome were selected to reconstruct the phylogeny. Total genomic DNA was extracted from frozen or alcohol preserved tissue sam-

ples of muscle or liver by digestion with proteinase K for 5-12 hr, and purified three times with phenol-chloroform-isoamyl alcohol (PCI), and then once with chloroform-isoamyl alcohol (CI). The mtDNA region of 12S through 16S was sequenced using the following method. Double stranded fragments were amplified in 33 cycles of the polymerase chain reaction (PCR; 92°C for 30 sec, 45-55°C for 30 sec 72°C for 1.5 min) performed in 25 µl reactions. Annealing temperatures were changed from 45°C to 55°C as needed in order to improve the quality of PCR products. Usually, PCR reactions amplified the entire fragment from 12S1L to 16S2H. Subsequently, several internal primers were used for sequencing. Infrequently, amplification of the larger fragment was not possible and thus the following primers were used: 12S1L, 12S2H, 16S3H, 16S3L, 16S5H, 16SML and 16S2H (Table 1). After amplification, the 25 l product was separated by electrophoresis on an agarose gel and stained with ethidium bromide. The bands containing DNA were excised and the DNA was eluted using Gene Clean II kit (Bio101) and suspended in distilled, deionized water. The cleaned DNA was sequenced directly with Thermo Sequenase 33P-labeled terminator cycle sequencing kit (Amersham). Locations of the primers are shown in Fig 1.

The products of the sequence reactions were resolved in a polyacrylamide 7M urea gel that was then dried and visualized on autoradiograph films (Kodak) within 24-48 hr. A few sequences were resolved using an ABI 377 automated DNA sequencer using the manufacturer's protocols.

DNA SEQUENCE ANALYSIS

Sequences were initially aligned using ClustalW (Thompson *et al.*, 1994) with gap-open and gap-exten-

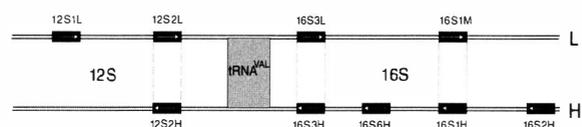


FIG 1. Schematic drawing of the 12S, tRNA^{Val}, and 16S mitochondrial gene and the relative positions of the primers used in this study. Hatched lines refer to primers that are complements to each other; L and H denote light and heavy strands, respectively. Specific primers are listed in Table 1.

TABLE 2. Summary of RNA genes sequenced from the ingroup and outgroup taxa. NT = Total number of taxa analyzed; TS = total number of homologous sites resolved; AS = number of ambiguous sites removed; NSR = number of homologous sites retained; NVS = number of variable sites; NPPIS = number of potentially phylogenetically informative sites; NMPTs = number of most parsimonious trees resolved; LMPTs = Length of most parsimonious solution; CI = consistency index; RI = retention index. Trees for the tRNAVal gene were not calculated (n/a) owing to the limited number of characters (37) available to resolve nodes among the 52 taxa in the analysis.

Gene	NT	TS	AS	NSR	NVS	NPPIS	NMPTs	LMPTs	CI	RI
12S rRNA	53	546	29	517	335	236	6	1664	0.276	0.524
tRNAVal	52	72	2	70	48	37	n/a	n/a	n/a	n/a
16S rRNA	69	1509	25	1484	910	728	16	6279	0.237	0.530
All RNAs	70	2127	56	2071	1301	1012	3	8512	0.239	0.527

sion penalties set to 5. Subsequently, minor adjustments to the computer alignments were made by eye in BioEdit (ver. 5.0.9; Hall, 2001) and MacClade (ver. 4.0.5; Maddison & Maddison, 2002). Sites with ambiguous alignment were excluded from the phylogenetic analysis because the homology cannot be confidently assumed (Hillis *et al.*, 1991). Only potentially cladistically informative sites were maintained for the analysis in PAUP* (ver. 4.0b8a; Swofford, 2001).

All multistate characters were evaluated as unordered because there is no *a priori* reason to assume order of evolutionary change between nucleotide bases adenine (a), cytosine (c), guanine (g), or thymine (t) (Swofford *et al.*, 1996). The phylogenetic analysis using PAUP* employed an heuristic search, with random addition sequence, 500 replicates, retaining minimal trees only, using tree bisection reconnection branch swapping with steepest descent and collapsing zero length branches. The two genes were initially analyzed separately, because different genes may experience different evolutionary pathways. Second, a combined data analysis was conducted. Ratios of transitions to transversions were calculated in MacClade.

Nodal support was assessed for the combined data sets. Bootstrap proportions (BSP; Felsenstein, 1985) used 1000 replicates calculated in PAUP*. We also performed decay analyses (DI; Bremer, 1988) using AutoDecay (ver 4.0.2; Eriksson, 1999).

RESULTS

Forty-five specimens were sequenced for 12S through 16S RNA genes. In total, 546 sites were sequenced for 12S, 72 for tRNAVal, and 1509 for 16S for a total of 2127 aligned sites. Among these sites, 56 were ambiguously aligned and 1012 were potentially phylogenetically informative (Table 2). All sequences were deposited in GenBank (12S = AF206072-AF206116; tRNAVal = AF206117-206161; 16S rRNA = 206453-206497).

PARSIMONY EVALUATION

For 12S, analysis of the 236 potentially informative sites yielded six most parsimonious trees (MPTs, Table 2). We did not attempt a separate phylogenetic analysis of the tRNAVal gene because there were too few potentially phylogenetically informative sites for a

meaningful analysis. For 16S, 728 potentially informative sites resulted in 16 cladograms, the differences constrained to one subclade. Because of similarities in nucleotide proportions and levels of site divergence, all RNA gene sequence data were combined for a total evidence analysis.

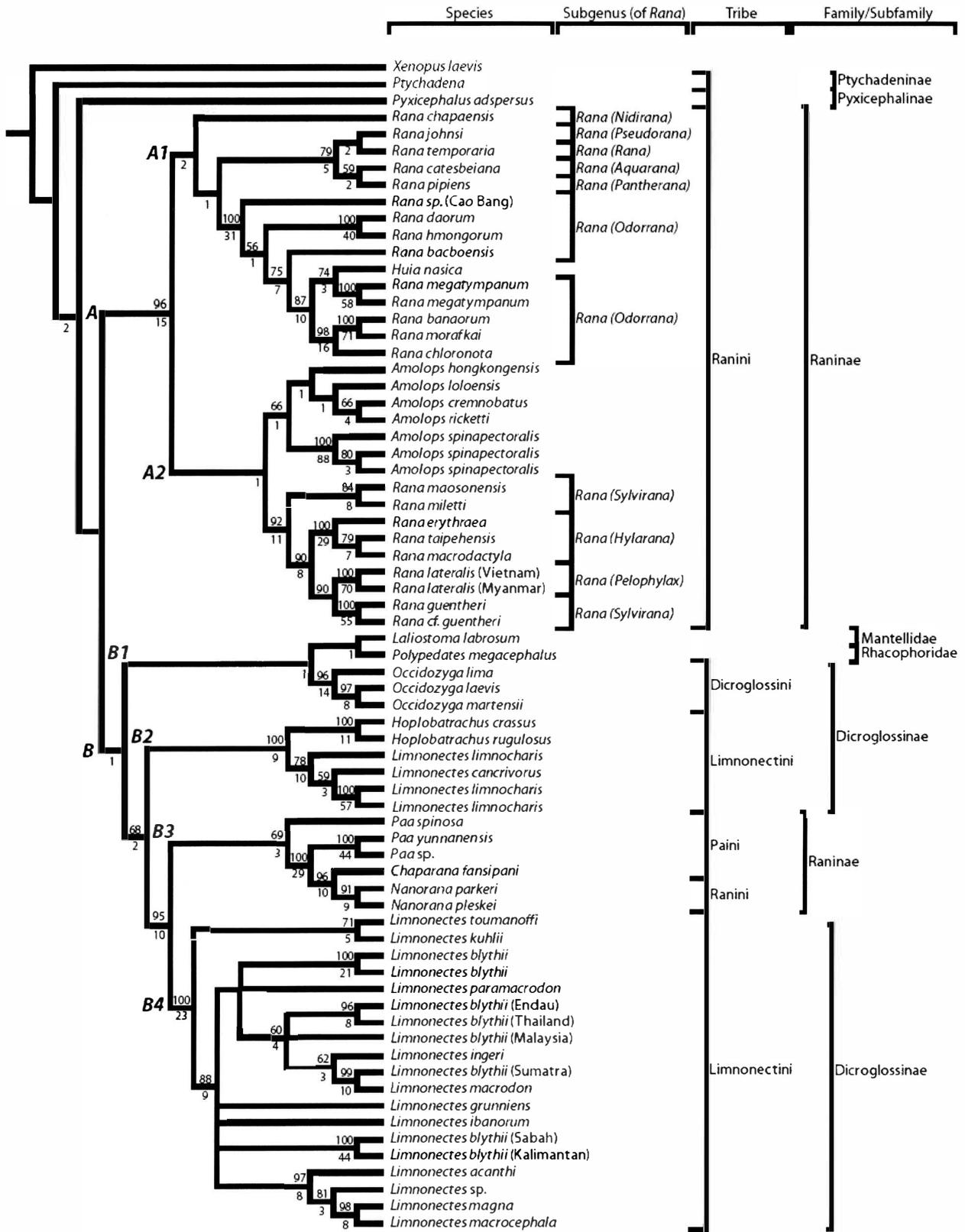
Combining all RNA gene sequence data into a single data set resulted in 1012 potentially cladistically informative characters. Analysis of these data yielded three most parsimonious trees (8512 steps in length, CI=0.24, RI=0.53). *Ptychadena* was resolved as the sister group of *Pyxicephalus* plus two major speciose clades (A and B) of *ranids* (Fig. 2): Clade A, included *Amolops*, *Hylarana*, *Nidirana*, *Odorrana*, and *Rana*; Clade B was composed of *Chaparana*, *Hoplobatrachus*, *Limnonectes*, *Nanorana*, *Occidozyga*, and *Paa*, plus a mantelline, *Laliostoma*, and rhacophorine, *Polypedates*.

Clade A was treated as having two major subclades, A1 and A2. *Nidirana chapaensis* was resolved as the sister group to all other members of clade A1, including specimens of *Odorrana* and *Rana*. *Rana (Pantherana) pipiens* was resolved as the sister species of *R. (Auarana) catesbeiana*. Their sister group contained *R. (Pseudorana) johnsi* plus *R. (Rana) temporaria*. The clade containing *Rana* formed the sister group to a clade composed of *Amolops (Huia) nasica* and a paraphyletic *Odorrana*.

In Clade A2, a monophyletic *Amolops (Amolops)* was the sister group to a clade of *Rana* including subgenera *Hylarana* and *Pelophylax*, and a paraphyletic subgenus *Sylvirana*.

Clade B was treated as having four major subclades, B1-B4. In Clade B1, the mantelline, *Laliostoma labrosum*, and the rhacophorine, *Polypedates megacephalus*, were resolved as sister taxa. Together they formed the sister group of the three species of *Occidozyga*. Clade B1, in turn, was the sister group of clades B2-B4. Clade B2 consisted of *Hoplobatrachus crassus*, *H. rugulosus*, *Limnonectes cancrivorus*, and a paraphyletic *L. limnocharis*. It was the sister group of clades B3 and B4. Clade B3 contained *Paa* (as a paraphyletic taxon), *Chaparana* and *Nanorana*. The sister group of Clade B3 was B4, which consisted of *Limnonectes blythii* and its relatives, with *L. blythii* being resolved as paraphyletic (Fig. 2.)

FIG 2. The strict consensus tree of the two most parsimonious explanations of mtDNA sequence data for south-east Asian ranids. *Xenopus laevis* was used to root the tree. Taxonomy reflects current usage. Taxonomic groupings proposed by Dubois, 1992 appear to the right of the tree. Numbers above the line are bootstrap proportions (50) and those below are Bremer decay indices examined up to six steps longer than the most parsimonious trees.



ASSESSING NODAL STABILITY

Values of nodal support are indicated on the tree (Fig. 2). Bootstrapping (BS) trials supported 43 nodes with a consistency greater than 70%. Decay analyses revealed that many nodes required a considerable number of additional steps to collapse, except those not generally supported by high BS proportions.

DISCUSSION

Because different portions of the mtDNA genome evolve at different rates, cladograms from different genes for the same set of organisms may differ. The relatively slowly evolving 12S and 16S rRNA genes seem appropriate for resolving older divergences, perhaps as old as 150 Ma (Mindell & Honeycutt, 1990).

The two mitochondrial genes evolved in similar ways. We assume that this conciliation is due to their phylogenetic history. Our phylogenetic analysis of the combined data resulted in three MPTs. Independent analyses for each gene revealed compatible branching patterns.

PREVIOUS STUDIES

The phylogenetic relationships of ranid frogs have been investigated in three recent molecular studies. Marmayou *et al.* (2000) evaluated a short, 305 bp segment of mtDNA 12S for 28 species of ranid and rhacophorid frogs using maximum parsimony, transversion weighting, and phenetic neighbour joining. Their unweighted parsimony evaluation resolved *Occidozyga* and *Phrynoglossus* as sister taxa, which together formed the sister group of all other ranids plus rhacophorids. The remaining taxa clustered into four groups whose relationships to each other were not resolved. Representative rhacophorids, including species of *Buergeria*, *Philautus*, *Polypedates* and *Chirixalus*, formed one monophyletic group. *Amolops* and *Rana chalconota* formed another group. The genera *Limnonectes*, *Fejervarya*, *Hoplobatrachus*, *Sphaerotecca*, and *Taylorana* formed a third clade and several species of *Rana* formed the fourth cluster. In this taxonomy, *Rana* was paraphyletic. Transversion weighting and the phenetic evaluation resolved paraphyly in *Philautus*, *Limnonectes*, and an additional example of paraphyly with respect to *Rana*. Given the small numbers of characters analyzed, it is not surprising that most nodes received low levels of branch support.

Bossuyt & Milinkovich (2000) evaluated 2692 bp of mitochondrial and nuclear homologous DNA sequence sites, excluding third position codon sites for cytochrome b. They constructed trees using maximum likelihood and BS consensus methods based on maximum parsimony. Because the initial outgroup was very divergent it was dropped from the analysis and Madagascar ranids and rhacophorids were used to root the network of Asian ranids and rhacophorids combined, and vice versa. Unfortunately, bootstrapping is problematic (Kluge & Wolf, 1993) and consensus methods

themselves have long been known to be suspicious (Miyamoto, 1985; Miyamoto & Fitch, 1995). Maximum likelihood analyses are philosophically problematic (Kluge, 1997; Siddall & Kluge, 1997). This puzzle is exemplified, in part, by "Brooks' conundrum" (D. R. Brooks, Univ. of Toronto, pers. comm., 2002): "Do you believe that evolution occurs in a most parsimonious manner? If not (which is demonstrably true-homoplasy exists), then why try to force a model of maximum parsimony on the analysis of your data, which is exactly what maximum likelihood does?" Maximum parsimony should be used as a criterion for selecting among all possible trees, and not as a model of evolution. Regardless, the basal relationships in the bootstrap consensus tree of Bossuyt and Milinkovich were unresolved. Asian treefrogs were monophyletic, as was a clade containing representative species of *Fejervarya*, *Hoplobatrachus*, *Nanophrys*, *Euphlyctis*, Asian *Tomopterna* (*Sphaerotecca*) and some *Limnonectes*.

Kosuch *et al.* (2001) investigated the monophyly of tiger frogs, *Hoplobatrachus*, which occur in both Asia and Africa. They evaluated 34 ranids using a total of 903 homologous nucleotide sites from 16SrRNA and 12SrRNA with 281 sites being potentially phylogenetically informative. Though their focus was on the biogeographical relationships of Asian and African *Hoplobatrachus*, representatives of *Fejervarya*, *Limnonectes*, *Nannophrys*, *Occidozyga*, *Phrynoglossus*, *Ptychadena* and several species of *Rana* were also included. Support was found for a monophyletic *Hoplobatrachus*, which was resolved as the sister group to *Fejervarya*. Subfamily Dicroglossinae was not resolved as a monophyletic grouping in either of the two trees presented.

More recently, Roelants *et al.* (2004) evaluated DNA sequences of several groups of ranid frogs, though their focus was on the biogeography of these frogs rather than taxonomy. The taxonomic implications of their study are summarized below.

PATTERNS OF RELATIONSHIPS

Although the relationships we resolved among the putative subfamilies of ranid frogs were not entirely consistent with previous taxonomies, lower taxonomic groupings were congruent in a number of ways with those proposed by Dubois (1986[1987], 1992). However, our analysis discovered several problematic associations. For example, the genus *Rana* was not resolved as a monophyletic taxon and *Limnonectes limnocharis* appears to be paraphyletic with respect to *L. cancrivorus*.

MONOPHYLY OF THE RANIDAE AND RELATIONSHIPS AMONG SUBFAMILIES

Family Ranidae was resolved as a paraphyletic taxon with respect to rhacophorids. Therefore, recognizing Family Rhacophoridae as a subfamily within family Ranidae, as suggested by Dubois (1992) and Blommers-Schlösser (1993), provides an acceptable solution.

Alternatively, in order to avoid having an extremely speciose Ranidae, multiple families could be recognized. The problem requires further investigation using sequences from more conserved genes and a broader array of taxa, especially African ranids and rhacophorids.

RANINAE, CLADE A

Clade A consisted of five potential genera of ranid frogs: *Amolops*, *Hylarana*, *Nidirana*, *Odorrana*, and *Rana*, although group membership did not mirror current taxonomy. These genera were distributed amongst two subclades (A1 and A2).

CLADE A1

Genus Rana (part), subgenus Nidirana: One species, *R. (Nidirana) chapaensis*, was used to represent this subgenus of *Rana*. It was resolved as the sister taxon to the following two subclades of Clade A1:

Genus Rana (part), subgenera Aquarana, Pantherana, Rana, and Pseudorana: One species each was used to represent four relatively speciose subgenera of *Rana*. The two North American species, *R. (Pantherana) pipiens* and *R. (Aquarana) catesbeiana*, were resolved as sister taxa. Their sister group contained the Asian species, *R. (Pseudorana) johnsi*, and its sister group represented by the European *R. (Rana) temporaria*.

Genus Rana, subgenus Odorrana: The group containing *Odorrana* and *Amolops (Huia)* forms the sister group to the clade containing *Rana catesbeiana* and *R. pipiens* plus *R. johnsi* and *R. temporaria*.

The large, odoriferous ranids sometimes referred to the genus *Odorrana* formed a paraphyletic lineage with respect to *Amolops (Huia) nasica*. *Amolops (Huia)* was resolved within a group of *Odorrana*, and not with other *Amolops* with which it is usually associated (Yang, 1991). *Amolops (Huia)* differs from *Odorrana* by its non-glandular skin and the absence of enlarged toe discs.

Our data also support the finding that *Odorrana chloronota* is a species complex (Murphy *et al.*, 1997; Bain *et al.*, 2003). As cryptic species are identified, the number of species of *Odorrana* will likely increase significantly.

In some regions, like the Khe Moi River, Nghe An Province, Vietnam, three large species of this clade occur in sympatry (Bain *et al.*, 2003). Some sympatric species are derived from distant lineages, such as the co occurrence of *O. chloronota* and a similar species, *O. bacboensis*. However, other sympatric species appear to be much more closely related, such as *O. chloronota* and *O. morafkai*. This pattern of sympatry repeats in most other areas in Vietnam, although the species composition changes.

CLADE A2

Genus Amolops, subgenus Amolops: the sampled species are monophyletic, and a larger survey of species

is currently underway. The two most anatomically similar species included in this analysis, *A. ricketti* and *A. torrentis*, formed a terminal sister relationship, followed basally by *A. loloensis*, and the geographically more distant, but anatomically similar *A. hongkongensis*. *Amolops spinapectoralis* was resolved as the sister group of these species. *Amolops* formed the sister group of the remaining subclade containing *Rana maasonensis* and *R. erythraea*.

Genus Rana, subgenera Hylarana, Pelophylax, and Sylvirana: this clade includes a paraphyletic assemblage of subgenera within the genus *Rana*. The association of subgenera is as follows: (*Sylvirana*((*Sylvirana*, *Pelophylax*)(*Hylarana*))).

RANINAE/RHACOPHORIDAE, CLADE B

The second major group of ranines contains relatively stocky, largely edible Asian frogs. Frogs within this clade belong to several genera, possibly reflecting, in part, their economic significance (and, hence, greater attention) and a greater amount of anatomical divergence. The frogs within clade B clustered into four serially arranged clades as follows: (B1, (B2, (B3, and B4))).

CLADE B1

Genera Occidozyga, Polypedates, and Laliostoma: The two representative rhacophorids, *Polypedates megacephalus*, a rhacophorine, and *Laliostoma labrosum*, a mantelline, were resolved as sister taxa. These taxa formed the sister group to a monophyletic Occidozyga. The sister group to this clade contains the diglossine frogs of the genera *Fejervarya*, *Hoplobatrachus*, and *Limnonectes*, separated by the ranine frogs *Chaparana*, *Nanorana*, and *Paa* (Fig. 2).

CLADE B2

Genera Hoplobatrachus, Limnonectes (part) and Fejervarya: This subclade, sometimes considered to be three genera, has been particularly problematic. Kosuch *et al.* (2001) examined the biogeographic relationships of *Hoplobatrachus*, and found a monophyletic *Hoplobatrachus* to be the sister group to *Fejervarya*. We also found a monophyletic *Hoplobatrachus*, with *H. crassus* plus *H. rugulosus* being the sister group to the remainder of the clade.

The rice frog, *L. limnocharis*, is resolved as paraphyletic with respect to *L. cancrivorus*. A considerable amount of allozyme work in other parts of its extensive range suggests that it is a composite of many cryptic species (e.g. Dubois, 1984; Toda *et al.*, 1994, 1998a,b). Our data and cladogram support this conclusion.

CLADE B3

Genera Paa, Chaparana, and Nanorana: This clade is a paraphyletic assemblage of genera. *Paa spinosa* is resolved as the sister group of a clade containing two other

species of *Paa* plus *Chaparana fansipani*, *Nanorana parkeri* and *N. pleskei*. Thus, the genus *Paa* is paraphyletic with respect to *Nanorana* and *C. fansipani*. The association of these species is particularly interesting, given that, though *Chaparana* and *Nanorana* are heavy-set, they are not large frogs like *Paa*. This association does not appear to be spurious since all nodes within this clade received substantial support. This clade, in turn, is resolved as the sister group to the remaining ranine clade.

CLADE B4

Genus Limnonectes (part): The third subclade of Asian edible frogs includes species placed in this genus. Within this clade, paraphyly is the rule rather than exception. Populations of *L. blythii* are variously associated with *L. macrodon*, *L. ingeri*, and *L. paramacrodon*. The clades have a greater correspondence to geographic location than taxonomy. Sister taxa co-occur on a single island. Some species appear to be large complexes of morphologically similar species. For example, Inger *et al.* (1999) noted several anatomical differences between *L. blythii* from the Malay Peninsula and southern Vietnam. Thus, as with *L. limnocharis*, the taxonomy of this group needs to be revised as it undoubtedly represents far more species than previously thought. Our arrangement differs from that of Roelants *et al.* (2004) who resolved this group as the sister of clade B2+B3. However, both studies found weak support at the conflicting nodes. Whereas we included 19 specimens, Roelants *et al.* (2004) sequenced two representatives.

TAXONOMIC IMPLICATIONS

Type species of Rana Linnaeus 1758: Before undertaking revisions, it is first necessary to establish the relationships of the type species of the genus *Rana*. Fleming (1822) designated *Rana temporaria Linnaeus*, 1758 as the type species of *Rana*. This species is the name-bearer of the genus, subgenus, tribe, subfamily, and family. Genus *Rana sensu* Frost (2004) has more than 240 species divided into 22 subgenera. It is one of the most speciose groups of vertebrates and contains many independent lineages. Taxonomically, recognition of these major lineages as genera would better summarize their phylogenetic history.

Taxonomic chaos: At virtually every hierarchical level, taxonomic problems exist. For example, tribe Ranini is a paraphyletic assemblage of genera with respect to the genus *Rana* and at the subfamilial level with the genus *Nanorana* Günther 1869.

The taxonomy of these frogs has been unstable. Not exhaustive, Table 3 briefly summarizes some of the changes for Asian groups from 1985 onward for many of the species included in this study. For most species, placement in one group or another has remained relatively stable, but the taxonomic rank accorded to the groups has been quite unstable. For example, the crab-eating frog, *Limnonectes cancrivorus*, was placed in

Genus *Rana*, subgenus *Euphlyctis* Fitzinger 1843 by Frost (1985), then into genus *Limnonectes* Fitzinger 1843, subgenus *Hoplobatrachus* Peters 1863 by Dubois (1986 [1987]). Subsequently, it was assigned to genus *Euphlyctis* by Fei *et al.* (1990), then to genus *Hoplobatrachus*, subgenus *Fejervarya* Bolckay 1915 by Dubois (1992). Most recently, the species was placed in Genus *Fejervarya* (Fei, 1999). Yet others (e.g. Inger, 1996; Nguyễn & Ho, 1996; Zhao, 1994; Zhao & Adler, 1993) have left the species in the genus *Rana*. Much of this taxonomic instability is due to the absence of a reasonable phylogeny upon which to identify membership within particular clades.

A phylogenetically based taxonomy reflects the greatest amount of information within a hierarchical system (Farris, 1967; Wiley, 1980; Brooks & McLennan, 1991, 2002). Below, we review the taxonomy of these frogs and make taxonomic changes that directly reflect phylogenetic history, albeit conservatively.

Subfamily Dicroglossinae, tribe Dicroglossini: This group was represented by three of 12 species from the genus *Occidozyga* Kuhl et Hasselt 1882: *O. laevis* and *O. lima*, and *O. martensii*. *Occidozyga laevis* and *O. martensii* have been placed in the genus *Phrynoglossus* Peters 1867 by many authorities (e.g., Peters, 1867; Smith & Chasen, 1931; Taylor, 1962; Dubois, 1986 [1987]). Our data do not refute this placement but recognition of *Phrynoglossus* could result in a paraphyletic *Occidozyga*.

Subfamily Raninae: The subgenus *Nidirana* Dubois 1992 contains seven species: *R. (Nidirana) adenopleura*, *R. (N.) caldwelli*, *R. (N.) chapaensis*, *R. (N.) daunchina*, *R. (N.) lini*, *R. (N.) pleuraden*, and *R. (N.) psaltes*. It has been resolved as the sister taxon to the clade containing *Rana temporaria* plus *Odorrana* Fei Ye et Huang 1991. Within the genus *Rana*, *R. johnsi* forms the sister group of the type species, *R. temporaria* and the two North American representatives of the subgenera *Pantherana* Dubois 1992 and *Aquarana* Dubois 1992. Our data neither refute recognition of Fei's genus *Pseudorana* Fei Ye et Huang 1991 nor support it. Given the lack of additional specimens from this group and arguments for its rejection (Tanaka-Ueno *et al.*, 1998), we believe it preferable not to recognize *Pseudorana* until sufficient evidence exists.

Genus Nidirana Dubois 1992: In order to maintain recognition of genus *Odorrana* and not render genus *Rana* paraphyletic, subgenus *Nidirana* must be elevated to generic status for *N. adenopleura*, *N. caldwelli*, *N. chapaensis*, *N. daunchina*, *N. lini*, *N. pleuraden*, and *N. psaltes*.

Genus Hylarana Tschudi 1838: The genus *Rana* is paraphyletic with respect to *Amolops* Cope 1865. In order to maintain the genus *Amolops*, another ranine genus must be recognized. The group of ranids that form the sister group of *Amolops* contains the subgenera *Hylarana* Tschudi 1838, *Pelophylax* Fitzinger 1843, *Sylvirana* Dubois 1992, and *Tenuirana* Fei Ye et Huang 1991. On the basis of priority, we recognize genus

TABLE 3. A representative summary of the history of names applied to some of the Asian species of ranid frogs investigated in this study.

Specific epithet	Frost 1985	Dubois 1987 “1986”	Fei <i>et al.</i> 1991 “1990”	Dubois 1992	Ye <i>et al.</i> 1993	Fei 1999
<i>hongkongensis</i>	<i>Amolops</i>	-	<i>Amolops</i>	<i>A. (Amolops)</i>	<i>Amolops</i>	<i>Amolops</i>
<i>loloensis</i>	<i>Amolops</i>	-	-	<i>A. (Amolops)</i>	-	-
<i>ricketti</i>	<i>Amolops</i>	-	<i>Amolops</i>	<i>A. (Amolops)</i>	<i>Amolops</i>	<i>Amolops</i>
<i>torrentis</i>	<i>Amolops</i>	-	<i>Amolops</i>	<i>A. (Amolops)</i>	<i>Amolops</i>	<i>Amolops</i>
<i>erythraea</i>	<i>R. (Hylarana)</i>	-	-	<i>R. (Hylarana)</i>	-	-
<i>guentheri</i>	<i>R. (Hylarana)</i>	-	<i>H. (Hylarana)</i>	<i>R. (Sylvirana)</i>	<i>H. (Hylarana)</i>	<i>Hylarana</i>
<i>nasica</i>	<i>Amolops</i>	-	<i>Amolops</i>	<i>A. (Huia)</i>	-	<i>Amolops</i>
<i>johnsi (as sauteri)</i>	<i>R. (Hylarana)</i>	-	<i>Pseudorana</i>	<i>R. (Pseudorana)</i>	<i>Pseudorana</i>	<i>Pseudorana</i>
<i>livida</i>	<i>R. (Hylarana)</i>	-	<i>Odorrana</i>	<i>R. (Eburana)</i>	<i>Odorrana</i>	<i>Odorrana</i>
<i>macroductyla</i>	<i>R. (Hylarana)</i>	-	<i>Hylarana (Tenuirana)</i>	<i>R. (Hylarana)</i>	<i>H. (Tenuirana)</i>	<i>H. (Tenuirana)</i>
<i>maosonensis</i>	<i>R. (Hylarana)</i>	-	-	<i>R. (Sylvirana)</i>	-	-
<i>milleti</i>	<i>R. (Hylarana)</i>	-	-	<i>R. (Sylvirana)</i>	-	-
<i>taipehensis</i>	<i>R. (Hylarana)</i>	-	<i>Hylarana (Tenuirana)</i>	<i>R. (Hylarana)</i>	<i>H. (Tenuirana)</i>	<i>H. (Tenuirana)</i>
<i>lateralis</i>	<i>Rana</i>	<i>Rana (Rana)</i>	-	<i>R. (Pelophylax)</i>	-	-
<i>pipiens</i>	<i>R. (Rana)</i>	<i>R. (Rana)</i>	-	<i>R. (Pantherana)</i>	-	-
<i>catesbeiana</i>	<i>R. (Rana)</i>	<i>R. (Rana)</i>	-	<i>R. (Aquarana)</i>	<i>R. (Aquarana)</i>	<i>R. (Aquarana)</i>
<i>fansiapani</i>	<i>Rana (Paa?)</i>	-	-	<i>C. (Chaparana)</i>	-	-
<i>kuhlii</i>	<i>R. (Limnonectes)</i>	<i>L. (Limnonectes)</i>	<i>Limnonectes</i>	<i>L. (Limnonectes)</i>	<i>Limnonectes</i>	<i>Limnonectes</i>
<i>toumanoffi</i>	<i>R. (Euphlyctis)</i>	<i>L. (Bourretia)</i>	-	<i>L. (Bourretia)</i>	-	-
<i>blythii</i>	<i>R. (Euphlyctis)</i>	<i>L. (Limnonectes)</i>	-	<i>L. (Limnonectes)</i>	-	-
<i>rugulosus</i>	<i>R. (Euphlyctis)</i>	<i>L. (Hoplobatrachus)</i>	<i>Tigrina</i>	<i>Hoplobatrachus</i>	<i>Hoplobatrachus</i>	<i>Hoplobatrachus</i>
<i>cancrivorus</i>	<i>R. (Euphlyctis)</i>	<i>L. (Hoplobatrachus)</i>	<i>Euphlyctis</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>Fejervarya</i>
<i>limnocharis</i>	<i>R. (Euphlyctis)</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>L. (Fejervarya)</i>	<i>Euphlyctis</i>	<i>Fejervarya</i>
<i>labrosa</i>	<i>Tomopterna</i>	<i>T. (Sphaeroteca)</i>	-	<i>T. (Sphaeroteca)</i>	-	-
<i>yunnanensis</i>	<i>R. (Paa) phrynoides</i>	<i>R. (Paa)</i>	<i>Paa (Paa) phrynoides</i>	<i>Paa (Gyandropaa)</i>	<i>Paa (Paa)</i>	<i>Paa (Paa)</i>
<i>parkeri</i>	<i>Altirana</i>	-	<i>Altirana</i>	<i>N. (Altirana)</i>	<i>Altirana</i>	<i>Nanorana</i>
<i>pleskei</i>	<i>Nanorana</i>	-	<i>Nanorana</i>	<i>N. (Nanorana)</i>	<i>Nanorana</i>	<i>Nanorana</i>
<i>chapaensis</i>	<i>Rana (Hylarana)</i>	-	-	<i>R. (Nidirana)</i>	-	-
<i>spinosa</i>	<i>R. (Paa)</i>	<i>R. (Paa)</i>	<i>Paa</i>	<i>Paa (Quasipaa)</i>	<i>R. (Paa)</i>	<i>Paa (Paa)</i>
<i>laevis</i>	<i>Occidozyga</i>	<i>Occidozyga</i>	-	-	-	-
<i>lima</i>	<i>Occidozyga</i>	<i>Phrynoglossus</i>	-	-	-	-

Hylarana. It contains those species associated with the subgenera *Hylarana*, *Sylvirana*, *Tenuirana*, and *Pelophylax*. The type species of *Hylarana*, *H. erythraea*, was included in our evaluation.

Recognition of the subgenera within *Hylarana* requires a phylogeny and the current taxonomy results in paraphyletic groupings (Fig. 2). For example, Dubois (1992) included *H. guentheri*, *H. maosonensis* and *H. milleti* in genus *Rana*, subgenus *Sylvirana*. However, whereas *H. guentheri* is the sister group of subgenus *Pelophylax*, *R. maosonensis* plus *R. milleti* is the sister group of the clade containing *R. guentheri* (subgenus *Sylvirana* in part), subgenus *Hylarana*, and subgenus *Pelophylax*.

The subgenus *Tenuirana* is also a puzzle. *Tenuirana* contains only *R. taipehensis* and *R. macrodactyla*. Although these two species are sister taxa, recognition of this subgenus results in the paraphyly of other subgenera. Thus, *Tenuirana* should not be elevated to generic status as it leaves *Hylarana* a paraphyletic taxon.

Given the large number of species in *Hylarana*, the apparent polyphyly within the subgenus *Sylvirana*, and the problems surrounding the recognition of *Tenuirana*, recognition of these or any other subgenera or genera is premature in the absence of a more complete phylogeny.

GENUS *RANA* LINNAEUS 1758

Rana temporaria is a member of the clade consisting of *R. johnsi* and the American frogs, *R. catesbeiana* and *R. pipiens*. Dubois (1992) included *R. johnsi* (as *R. sauteri*) in the subgenus *Pseudorana*, and *R. pipiens* in subgenus *Pantherana*. He placed *R. temporaria* in subgenus *Rana*, and *R. catesbeiana* in subgenus *Aquarana*. This subgeneric arrangement is phylogenetically acceptable from the perspective of our data. Taxonomically, these species have been closely associated with one another.

Genera Odorrana Fei Ye et Huang 1991 and Huia Yang 1991: The usually large, odoriferous frogs referred to the genus *Odorrana* are the sister group to *Rana*. The type species for the genus *Odorrana* is *Rana margaretae* Liu, 1950 by original designation. Unfortunately, we did not have tissue samples from this species and no sequences exist in GenBank. Nevertheless, for the moment, we recognize genus *Odorrana* and include within it *O. bacboensis*, *O. banaorum*, *O. chloronota*, *O. daorum*, *O. hmongorum*, *O. megatympanum*, *O. morafkai*, and *O. nasica*. This list of species is not exclusive and at least 13 additional species could belong to the genus, including: *O. andersonii*, *O. anlungensis*, *O. exiliversabilis*, *O. grahami*, *O. hainanensis*, *O. jingdongensis*, *O. huangwuensis*, *O. livida*, *O. lungshengensis*, *O. margaretae*, *O. nasuta*, *O. schmackeri*, and *O. swinhoana*.

Genus Amolops Cope 1865: Few have questioned the validity or membership of genus *Amolops*, though our data reveal that *Amolops (Huia) nasica* occurs within the clade containing *Odorrana chloronota*. Con-

sequently, membership in one genus or another may be uncertain for many of the larger species referred to as either *Amolops (Huia)* or *Odorrana* (see above).

Subfamily Limnonectinae (new content/combination): Dubois placed genus *Paa* Dubois 1975 in subfamily Raninae, tribe Paini. However, subfamily Raninae is a paraphyletic group. Consequently, tribe Paini must be moved from subfamily Raninae and placed in subfamily Dicroglossinae, tribe Limnonectini along with the genera *Hoplobatrachus* and *Limnonectes*. However, doing so still leaves subfamily Dicroglossinae a paraphyletic group with respect to the Rhacophorinae and Mantellinae. Thus, to avoid paraphyly, tribe Limnonectini must be elevated to subfamily Limnonectinae. Recognition of the families Rhacophoridae and Mantellidae will necessitate recognition of the family Limnonectidae.

Limnonectinae has three distinctive lineages (Fig. 3). One lineage contains genus *Hoplobatrachus* and some species of genus *Limnonectes* referred to genus *Fejervarya* by Fei (1999). These frogs are placed in the tribe Hoplobatrachini (new combination). Another lineage, tribe Paini, contains the genera *Chaparana* Bourret 1939, *Nanorana* and *Paa* (but see below). Finally, tribe Limnonectini contains genus *Limnonectes* excluding those species previously referred to *Fejervarya*.

Genus Hoplobatrachus Peters 1863: This genus was represented by the species *H. crassus* and *H. rugulosus*. Kosuch *et al.* (2001) found this genus to be monophyletic. Our data support their conclusion.

Genus Fejervarya Bolckay 1915: *Fejervarya* is represented, in this clade, by the two species *F. limnocharis* and *F. cancrivora*. However, *F. limnocharis* is paraphyletic with respect to *F. cancrivora*. Though both species are generally assigned to genus *Limnonectes*, Fei (1999) included both species in the genus *Fejervarya*. Our data and cladogram support this conclusion.

Genera Chaparana Bourret 1939, Nanorana Günther 1896, and Paa Dubois 1975: The subclade containing *Paa* also contains members of the genera *Chaparana* and *Nanorana*. The genus *Paa* contains more than 29 species (Frost, 2004) of which two were included in our study plus one undescribed species. The genus is paraphyletic. The genera *Nanorana* and *Chaparana* fall out as sister taxa within the genus *Paa*. Among the available generic names, *Nanorana* (type species *N. pleskei* by original designation) is the oldest available name having priority over *Altirana* Stejneger, 1927 (type species *N. parkeri* by original designation), *Chaparana* (type species *Rana (Chaparana) fansipani* by original designation), and *Paa* (type species *Rana liebigii* Günther, 1860, [named originally as a subgenus of *Rana*] by original designation). Paraphyletic relationships preclude retention of the subgenera within *Nanorana*. In addition to species already included in the genus *Nanorana*, we add those species previously recognized as *Paa*, as well as *Nanorana fansipani*, *Nanorana aenea*, *N. delacouri*, *N. quadranus*, *N.*

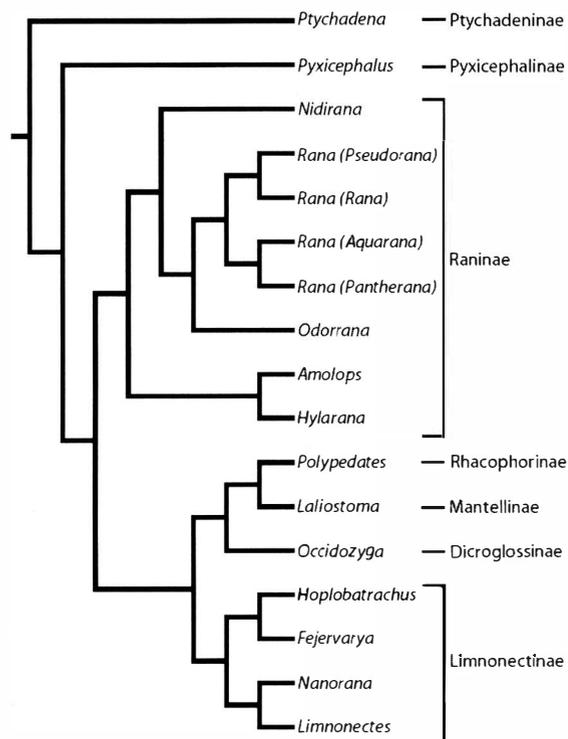


FIG 3. A reduced cladogram with genera as terminal taxa depicting nomenclatorial adjustments. To the right of the tree are the subfamilial taxonomic groupings supported by this study. Taxonomy reflects the recommendations of this manuscript.

sikimensis, *N. unculuanus*, *N. parkeri*, *N. pleskei* and *N. ventripunctata*.

Genus Limnnectes Fitzinger 1843: The type species of *Limnnectes* is *L. kuhlii* by original designation. We recognize *Limnnectes* for the following species included in our study, *L. acanthi*, *L. blythii*, *Limnnectes* sp. (“*duboisii*”), *L. grunniens*, *L. ibanorum*, *L. ingeri*, *L. kuhlii*, *L. macrocephala*, *L. macrodon*, *L. magna*, *L. paramacrodon*, and *L. toumanoffi*, and exclusive of *Fejervarya limnocharis* and *F. cancrivora*. The tree of Roelants *et al.* (2004) does not conflict with this new taxonomy.

Although our analysis contains a small number of ranid frogs, major Asian groups are represented herein. No doubt the genus *Rana* remains a “megataxon” in that it is a paraphyletic assemblage of species. Our evaluation revealed that most assemblages of species contained paraphyletic grades of species, and not monophyletic assemblages. Consequently, in the interest of nomenclatorial stability we believe that further divisions of ranid frogs in the absence of a phylogenetic hypothesis will only result in additional confusion in an already incredibly complex history of names and species. We have initiated further biochemical studies on some genera, particularly *Amolops*, *Odorrana* and *Paa*, but also including Vietnamese species in the genus *Hylarana*. Future investigations using gene sequences from 12S and 16S rRNA of smaller subsets of species should

prove equally fruitful for resolving relationships among the genera of ranid frogs.

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THE EFFECTS OF SHELTER AVAILABILITY AND SUBSTRATE QUALITY ON BEHAVIOUR AND POST-METAMORPHIC GROWTH IN THREE SPECIES OF ANURANS: IMPLICATIONS FOR CAPTIVE BREEDING

PATRICK T. WALSH AND J. ROGER DOWNIE

Institute of Biomedical and Life Sciences, Division of Environmental and Evolutionary Biology, University of Glasgow, Glasgow, UK

Growth rate strongly influences survival and reproductive success in anurans, particularly during larval and juvenile stages. In tadpoles the availability of shelter has been linked to increased growth rates, but work on recently metamorphosed anurans has been limited. Three species (*Physalaemus pustulosus*, *Leptodactylus fuscus* and *Mannophryne trinitatis*) were used to examine the effects that shelters have on growth rates and behaviour in the laboratory. Shelter availability had a strong effect on growth in *M. trinitatis* and a weaker effect on *L. fuscus* and *P. pustulosus*. Shelter provided advantages in the trade-off between predator avoidance and resource gathering and/or osmoregulatory benefits. Osmoregulatory benefits may have had the greater impact on growth rates for three reasons: (1) the ability to burrow in *L. fuscus* and *P. pustulosus* would more likely offset the osmoregulatory benefits of having shelters available, as individuals without shelters spent significantly more time burrowing; (2) individuals with shelter available were more active than those without, but the difference was not significant; and (3) the humidity under the shelters was significantly greater than within the rest of the tank. All frogs spent a large amount of time under shelters, if available. Regardless of the causes for any accelerated growth rate, shelters should therefore be provided in any terrarium for captive breeding colonies. The substrate choice of all three species reflected their natural habitat, with *M. trinitatis* (the stream frog) spending the majority of time in water and the toad-like *P. pustulosus* spending < 5% of its time in water. *L. fuscus* behaviour also reflected its natural habitat preferences. Thus, careful consideration of a species' natural history must be made when selecting the substrates to be used in terraria.

Key words: dendrobatids, juvenile anurans, leptodactylids, osmoregulation, refuge use

INTRODUCTION

Growth rate is an important factor for survival in amphibians (Bardsley & Beebee, 2001) as mortality rates are highest in the very young, predominately due to their small size making them more susceptible to predators. The amount of time it takes individuals to grow to a less vulnerable size has a huge impact on the survival of a clutch (Clarke, 1974). Size has also been shown to have consequences for reproductive success (Ryan, 1980). Female mate choice, in addition to being based on absolute size, has been shown to be influenced by juvenile growth rates in males (Halliday & Verrell, 1988). Consequently, it is important to examine factors that could affect growth rates in anuran life stages. In many animals, the risk of predation is a key factor affecting growth, since foraging activity is reduced in the presence of predators (Babbitt, 2001; Babbitt & Jordan, 1996; Relyea & Werner, 1999; Anholt & Werner, 1995; Skelly & Werner, 1990). This reduction of activity can be advantageous, since prey movement increases the ability of predators to identify and locate them (Lima & Dill, 1990; Werner & Anholt, 1993), but can also result in a trade-

off between growth rate and predation risk. Lima & Dill (1990) reviewed work on many taxa showing the costs of anti-predator activities and attributes such as predator avoidance, predator vigilance, flight response and toxin production. Although there are several strategies and extents to which they are used, an important tactic to avoid predation is the use of refuges or shelters (Sih, 1997). Shelter confers protection by making prey less visible and less accessible to predators. In an open environment a species without a high degree of toxicity may have to remain relatively inactive to avoid detection by predators, which often use movement as a cue. Conversely, if shelters are available the animal has a secure location where it can avoid predators. This can allow the animal to forage more since it can accurately gauge its response to predators, maximising its time spent foraging and minimising wasted energy from fleeing for long distances or unnecessarily (Ydenberg & Dill, 1986). In addition to the advantages conferred by escaping predation costs, shelters could allow most post-metamorphic anurans to increase their ability to capture prey, since most frogs, with the exception of the dendrobatids, are ambush predators. Furthermore, the availability of shelters may reduce osmoregulatory and thermal stresses (Hoffman & Katz, 1989), which can also influence growth rates (Seebacher & Alford, 2002).

Correspondence: J. R. Downie, Division of Environmental and Evolutionary Biology, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, UK.
E-mail: J.R.Downie@bio.gla.ac.uk

The majority of anuran research is focused on the ecology and biology of the larval and adult life stages, which are typically concentrated around bodies of water and are thus conspicuous and easy to sample. Thus, little emphasis has been placed on the juvenile stage, between metamorphosis and sexual maturity. Like the larval stage, the juvenile stage represents a critical period since individuals are highly vulnerable to predators (Babbitt & Tanner, 1998), and it spans a substantial portion of most anurans' life history, often being substantially longer than the tadpole stage. Therefore, factors affecting growth and survival during this stage will have huge impacts on adult populations. There have been several studies on the effects of shelter availability and predation on growth rates in tadpoles (Babbitt & Tanner, 1998; Relyea & Werner, 1999) and post-metamorphic growth rates in general (Clarke, 1974; Labanick & Schlueter, 1976), but few on the effects of shelter availability or predation on juvenile growth rates.

Reintroductions have recently been utilized to halt the decline of amphibian species and populations, but have had mixed results (Dodd & Seigel, 1991; Bloxam & Tonge, 1995; Burke, 1991), with failures being attributed to poor planning and monitoring. Most key life-history traits of amphibians, such as high fecundity and low maintenance costs, make them prime candidates for relocation, repatriation, and translocation (RRT) programmes. However, one important aspect of any successful amphibian reintroduction programme is establishing a captive-bred population capable of quickly producing surplus individuals for eventual release. Therefore, it is important to find the best method to rear high quality individuals to establish breeding colonies of any potential candidate for RRT programmes. Although none of the three species utilized in this study are endangered or being considered for any current RRT programme, knowledge of their behaviour and growth rates under laboratory conditions will provide valuable insights into general juvenile anuran responses to shelter availability. Such insights could be applied to future amphibian captive-breeding programmes, to establish captive breeding colonies or determine sites with suitable cover to facilitate rapid growth in released individuals.

In this study we examined the growth performance of juvenile individuals of three species with and without shelters provided, to determine if shelter influenced growth rates. Additionally, behavioural responses to the availability of shelters under normal and disturbed conditions were examined to test anti-predator behaviour. These allowed an assessment of whether the availability of shelter facilitates a higher growth rate and determined possible causes related to predator avoidance or osmoregulatory benefits. Finally, the substrate that the frogs spent most of their time on was examined to test whether there was a preference for any one type, to aid in providing an understanding of how the substrates in terraria should be determined.

METHODS AND MATERIALS

SPECIES AND STUDY DESIGN

The three species used were *Physalaemus pustulosus* (Cope), *Mannophryne trinitatis* (Garman) and *Leptodactylus fuscus* (Schneider). All specimens were collected from the wild in Trinidad, West Indies during August 2002, either as spawn or as tadpoles. Tadpoles were reared at Glasgow University at low density and began metamorphosing in early September. Prior to the start of the study the froglets of all species were maintained on *Drosophila* in stock tanks until the experiment began in early October.

P. pustulosus is a small, terrestrial, non-toxic toad-like leptodactylid (males: 28 mm; females: 32 mm) that inhabits savannah areas as well as being a human commensalist (Netting, 1930). Metamorphosis in this species can occur as soon as three weeks after oviposition, depending on rearing conditions (Downie, unpublished observations). *P. pustulosus* is known to be preyed upon by the manioc crab (*Pseudothelphusa garmani*) and fringe-lipped bats (*Trachops cirrhosus*).

L. fuscus is a medium-sized (males: 42 mm; females: 50 mm), terrestrial, non-toxic leptodactylid found in forests and savannahs, but mainly in association with large temporary and/or permanent pools (Kenny, 1969; Hoogmoed & Gorzula, 1979). Metamorphosis occurs as soon as three weeks after oviposition, depending on rearing conditions (Downie, unpublished observations), unless interrupted by dry conditions that can cause the larvae to enter a state of arrested development (Downie, 1984).

M. trinitatis is a small (males: 19-22 mm; females: 22-26 mm), diurnal, terrestrial, non-toxic dendrobatid found in forests or along forest-edges and uses stream and stream-edge habitats to forage among near-by leaf-litter for small insects (Murphy, 1997). Metamorphosis takes place as soon as four weeks after oviposition, depending on rearing conditions (Downie, unpublished observations). Snakes are common predators of *M. trinitatis*, particularly *Liophis reginae zweifleri* (Test *et al.*, 1966; Kenny, 1979). Murphy (1997) lists the history of the taxonomic revisions of this species, which have led to the recent creation of the genus *Mannophryne*.

This study was run under laboratory conditions (constant temperature of 27°C, 65-75% humidity and 12:12 LD schedule) similar to natural conditions in Trinidad's wet season, June to December (Beard, 1946; Granger, 1982). Thirty Perspex tanks (10 cm × 20 cm × 10 cm; ten tanks for each species) were set up in the same manner, each with three separate substrates: gravel, water and sand, and covered in muslin, held in place by elastic bands. Water was held in open Petri dishes in the centre of each tank, with the sand and gravel at either end. Water was added to the sand and gravel to keep them moist. Each of the substrates covered approximately one-third of the tank base, and about 1 cm deep, to allow enough depth for the frogs to burrow. In half of the

tanks (five per species) three PVC shelters, constructed from piping cut into 4 cm arcs of 4 cm height, were added so that a shelter was over a portion of each of the substrates. The shelters were arranged in a way that allowed the frogs to be visible at all times, even when underneath a shelter. Two frogs of similar size were chosen randomly from the stock population of each species and placed together in a tank, so that there were 20 frogs of each species, ten for each treatment (shelter; no shelter) and several individuals remaining in the stock tanks in the event of deaths. Only two individuals were placed in each tank so that individuals could be located and identified easily and to reduce competition for food. For measurement purposes, individuals in each tank were differentiated by slight differences in snout-vent length or pattern differences.

All three species were fed live fruit flies (*Drosophila melanogaster*), obtained from the University's Division of Molecular Genetics and contained in plastic tubes attached to the side of the tanks, so that no additional shelter was provided. All tanks were given tubes with approximately the same quantity of flies, and tubes and water were replenished six days a week during the study, so that there was always an excess of flies and constant moisture levels in the Petri dishes and substrates. The location of the *Drosophila* tubes was changed weekly between five locations, determined randomly by rolling a die (rolls of six were ignored), in order to limit the impact food source position had on any substrate preference. After eight weeks, the diet of *L. fuscus* was changed to silent crickets (*Gryllus assimilis*), obtained from Peregrine Livefoods weekly and split evenly between the ten tanks.

All individual frogs were weighed and measured when initially placed into the experimental tanks and reweighed and measured twice a week for eight weeks, with the exception of *M. trinitatis* (see Results). Additional data were collected from *L. fuscus* for five weeks on the diet of crickets. Mass was measured on a digital balance to 0.001 g after the frogs had been quickly surface-dried in a paper towel. Length measurements were taken from the tip of the snout to the vent (SVL) with callipers accurate to 0.1 mm. Although difficult to determine accurately (Turner, 1960), SVL was used in this study since it requires less handling than the measurement of tibia length described by Clarke (1974). Accuracy was increased by holding the dish vertically, forcing the frogs to adopt a position where they could adhere to the side of the dish: this was less variable than the frogs' sitting position. Measurements were repeated two to three times, until repetition or a median measurement was established. Body condition, calculated from the equation proposed by Veith (1987), was assessed, but did not indicate any clear trends and will not be presented.

Behavioural observations, taken on different days from growth measurements, were made at roughly the same time in the afternoon to avoid the effects of any diel variations in behaviour. On the days behavioural

observations were being carried out, feeding was always carried out after the observations were made. Each frog was observed for one minute; the substrate the frog was on, including the walls, and the activity of the frog were noted at three-second intervals, so that twenty readings were taken for each frog each day. The activity of the frogs was categorised as sitting, feeding, turning, sheltering or jumping. The 'turning' category encompassed all movements that were not jumping, such as slight positional changes. Whether the frog was burrowed and/or under a shelter was also noted. The effect of disturbance was also examined by erratically moving the tanks and removing the muslin cover, to elicit an anti-predator response. This was carried out on each tank prior to the second weekly observations, with the exception of the first two weeks.

After all the frogs were removed from the study tanks, two tanks were retained to measure humidity under the shelters and within the tank at large. The relative humidity was measured using an electronic thermo-hygrometer (Oregon Scientific) accurate to 1% RH, on each substrate both under the shelter and as far from the shelter as possible. A total of twelve readings were taken for each substrate in both tanks.

STATISTICAL ANALYSIS

Regression analysis was used to determine the rate of change over time of body weight and SVL for each species of frog. The slopes of the regression equations from the group with shelter available and those without shelter available were compared using a slope *t*-test. For the behaviour data the mean time on each substrate or activity was calculated for each tank for each day of observations. To determine if shelter availability or disturbance influenced substrate preference or activity levels in *P. pustulosus* and *L. fuscus*, multivariate analysis was carried out (on arcsin transformed data in *P. pustulosus*). ANOVA was used, with a post-hoc Tukey test, to determine differences between preferences in substrates. If the two treatments did not influence the substrate preference then a mean was taken for each tank so that the ANOVA was performed with one value for each substrate per tank. Due to the small number of observations, in *M. trinitatis* parametric tests could not be used so all substrate data were combined and analysed using the Kruskal-Wallis test and two chi-squared tests to determine the influence of shelter and disturbance on activity levels. A Mann-Whitney test was used to determine any difference in the time spent in a burrow by frogs when shelters were available or unavailable and if there was a difference between the relative humidity within the tank and under the shelters.

RESULTS

MORTALITY

There were no *P. pustulosus* deaths during the entire study. In *L. fuscus* there was one death and one probable escape. The death occurred in the second half of week 3;

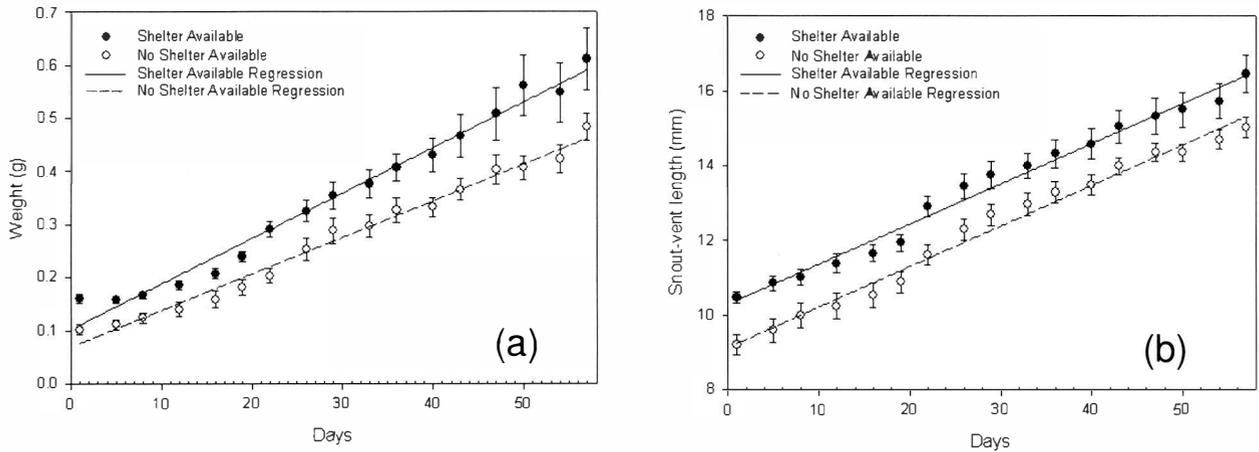


FIG. 1. (a) Mean mass \pm SE of *P. pustulosus* with and without shelter, with regression lines. Shelter: Mass = $0.103 + 0.00854$ days, $r^2 = 98.3\%$. No shelter: Mass = $0.0693 + 0.00687$ days, with $r^2 = 98.6\%$. (b) Mean length \pm SE of *P. pustulosus* with and without shelter, with regression lines. Shelter: SVL = $10.3 + 0.11$ days, $r^2 = 98.3\%$. No shelter: SVL = $9.13 + 0.11$ days, with $r^2 = 98.5\%$.

however, the individual had appeared moribund at the first weekly measurement, so a similar sized individual from the stock tank was measured as a potential replacement. Since the measurements of the replacement individual were taken on the same day as the study individual it was possible to extrapolate measurements for the replacement for previous weeks, by calculating the percentage of change per week in the original individual and subtracting those percentages from the measurements from the new individual taken at the beginning of the week, allowing continuity to be maintained. The probable escape occurred in the final week. The final measurements were obtained by calculating the mean percentage increase in the final week of individuals in the same treatment group and increasing the week 13 measurements of the individual by that percentage.

From the outset of the study *M. trinitatis* had very high mortality rates, with five deaths during the first week and ten in the second week (37.5% weekly mortality rate for the first two weeks). There was no difference

in the rate of mortality between the shelter and non-shelter treatments ($U=29.5, P> 0.05$). In all instances of death in the first two weeks the dead individuals were removed and replaced with individuals of similar size from the stock tank. After the two weeks all individuals from the stock tank had been used for the study and with four more deaths in week 3 there were not enough individuals remaining to provide sufficient replication. Therefore the remaining individuals were left undisturbed, other than for feeding and watering, in the study tanks. During the final five weeks, there were only eight deaths (5.7% weekly mortality rate). There were still eight individuals alive when the study finished, only six of them frogs that were originally placed in the study tanks in the first week: three in each treatment group. All available data on these individuals were used to give a measure of the growth rates under the two treatments; behavioural data from all individuals were analysed since we were not comparing the behaviour of individual frogs.

TABLE 1. Difference in growth rate between sheltered and non-shelter groups in each of the three species. For *L. fuscus*, Phase 1: 0-26 days; Phase 2: 29-57 days; Phase 3: 61-91 days.

Species			<i>t</i>	df	<i>P</i>
<i>P. pustulosus</i>	Mass		4.69	30	<0.005
	SVL		0.68	30	>0.05
<i>L. fuscus</i>	Mass	Initial 9 weeks	0.96	30	>0.05
		Total 14 weeks	0.49	42	>0.05
		Phase 1	5.85	8	<0.001
		Phase 2	5.57	14	<0.005
		Phase 3	3.88	8	<0.01
	SVL	Initial 9 weeks	3.01	30	<0.005
		Total 14 weeks	4.28	42	<0.001
Phase 1		4.51	8	<0.005	
<i>M. trinitatis</i>	Mass	Phase 2	0.40	14	>0.05
		Phase 3	4.30	8	<0.005
	SVL		4.17	8	<0.005
			2.21	8	<0.05

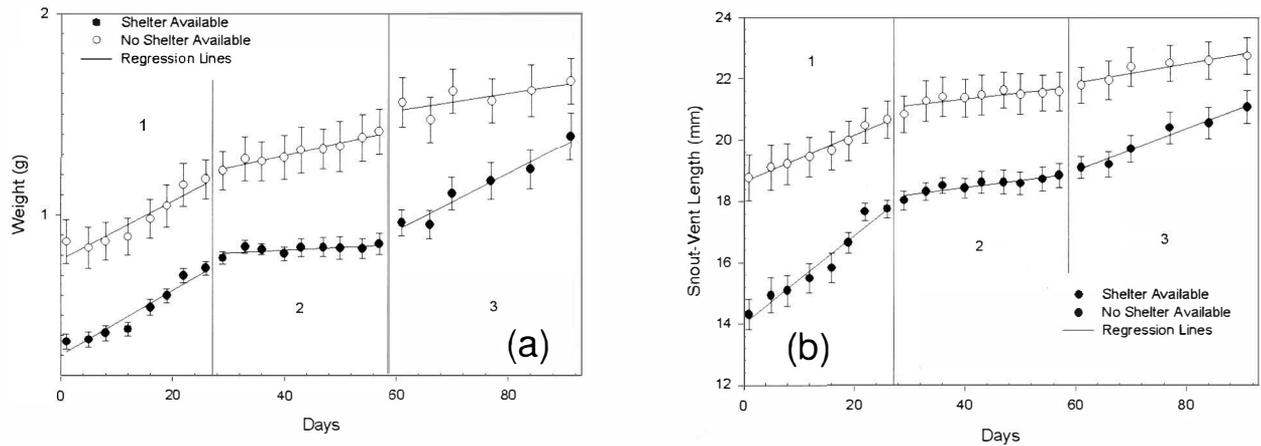


FIG. 2. (a) Mean mass \pm SE of *L. fuscus* with and without shelter, divided into three segments, with regression lines. The slopes of the three segments (ordered from left to right) for the sheltered group are: $b_1=0.016$, $b_2=1.39 \times 10^{-3}$, $b_3=0.014$. The slopes for the non-shelter group are: $b_1=0.0145$, $b_2=6.04 \times 10^{-3}$, $b_3=4.21 \times 10^{-3}$. (b) Mean SVL \pm SE of *L. fuscus* with and without shelter, divided into three segments, with regression lines. The slopes of the three segments (ordered from left to right) for the sheltered group are: $b_1=0.144$, $b_2=0.023$, $b_3=0.068$. The slopes for the non-shelter group are: $b_1=0.076$, $b_2=0.020$, $b_3=0.03$.

TABLE 2. Difference in starting weight and SVL between the two shelter treatments for each of the three species.

Species		<i>t</i>	<i>U</i>	df	<i>P</i>
<i>P. pustulosus</i>	Mass	4.68		17	<0.001
	SVL	4.06		17	<0.005
<i>L. fuscus</i>	Mass	4.30		11	<0.005
	SVL	5.03		15	<0.001
<i>M. trinitatis</i>	Mass		10.0		>0.05
	SVL		9.5		>0.05

GROWTH RATE

P. pustulosus increased in mass faster when shelters were present; however, there was no difference in SVL growth rate between the two treatment groups (Fig 1a, b; Table 1). There was a significant difference in starting mass and SVL of the two treatment groups (Table 2).

During the initial nine-week period the rate of increase in the mass of the *L. fuscus* group with shelters was not significantly different from the rate of increase

in the group without shelters (Fig 2a). However, *L. fuscus* individuals grew faster in SVL when shelters were present (Fig. 2b; Table 1). There was a difference between the groups with and without shelters in starting mass and SVL (Table 2).

When the data collected during the additional five weeks were included the results were similar, with no difference in the increase in mass, but a difference in SVL growth rate. However, it was clear that the growth rate of *L. fuscus* was not constant (Fig 2a,b; Table 1). There were three separate phases of growth for both groups; early growth (days 0-26), a plateau (days 29-57) and finally growth again (days 61-91) when fed on the diet of crickets.

There was a difference in the rate of increase in weight between the two groups during all three growth phases. The group without shelters increased in mass at a faster rate during the second stage, but the reverse was true of the first and third growth phase. When split into the three stages there was a significant difference in the rate of increase in SVL in the first, which was more dramatic than when considering growth as one continuous

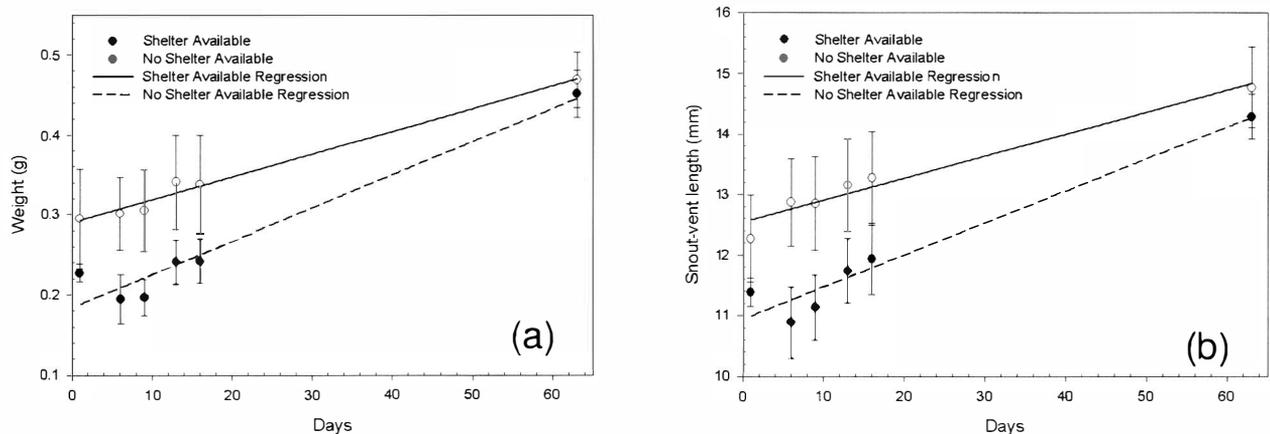


FIG. 3 (a) Mean mass \pm SE of *M. trinitatis* with and without shelter, with regression lines. Shelter: Mass = $0.184 + 0.00415$ days, $r^2 = 94.7\%$. No shelter: Mass = $0.290 + 0.00286$ days, $r^2 = 98.4\%$. (b) Mean SVL \pm SE of *M. trinitatis* with and without shelter, with regression lines. Shelter: SVL = $10.9 + 0.05$ days, $r^2 = 94.3\%$. No shelter: SVL = $12.5 + 0.04$ days, $r^2 = 95.8\%$.

TABLE 3. Percentage time spent burrowed by each species, with and without shelters (means ±SE). For the results of Mann-Whitney test (*U*): **P*<0.05 ** *P*<0.005.

	Shelters available	No shelters available	<i>U</i>
<i>P. pustulosus</i>	7.4±3.9%	29.6±5.6%	143**
<i>L. fuscus</i>	12.0±3.6%	38.3±8.7%	75*
<i>M. trinitatis</i>	3.8±3.8%	1.9±1.9%	104.5 NS

process. The second stage did not show any difference, but individuals increased in SVL faster in the third growth phase when shelters were present (Table 1).

M. trinitatis individuals grew significantly faster in mass (Fig 3a) and length (Fig 3b) when shelters were provided (Table 1). There was no significant difference in the starting mass or SVL (Table 2) of the two treatment groups.

BEHAVIOUR

Habitat choice. If shelters were available both *P. pustulosus* and *L. fuscus* spent a majority of the time under them. However, *M. trinitatis* spent only half as much time under the shelters as the other species (mean ±SE: 37.9±10.4%). Additionally, in both *P. pustulosus* and *L. fuscus*, individuals spent more time under the shelters when disturbed compared to undisturbed, but the differences were not significant (Fig. 4). When shelters were provided, *P. pustulosus* and *L. fuscus* spent less time burrowed than in the absence of shelters. However, *M. trinitatis* spent very little time burrowed and unlike the other two species, the sheltered group spent marginally, but not significantly, more time burrowed (Table 3).

SUBSTRATE USE

In both *P. pustulosus* and *L. fuscus* disturbance or shelter availability had no influence on which substrate was preferred, and all three species showed significant preferences for particular substrates over others (Fig. 5; *P. pustulosus*: $F_{3,36}=26.16, P<0.01$; *L. fuscus*: $F_{2,27}=6.35, P<0.01$; *M. trinitatis*: $H=29.2, df=3, P<0.001$). *P. pustulosus* showed a preference for the ter-

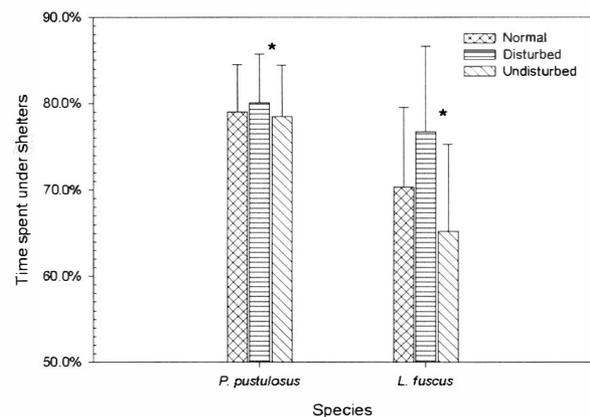


FIG. 4. Mean time spent under shelters in total and under disturbed and undisturbed conditions. Results of Mann-Whitney *U*-test for differences between disturbed and undisturbed conditions +SE bars (**P*<0.05).

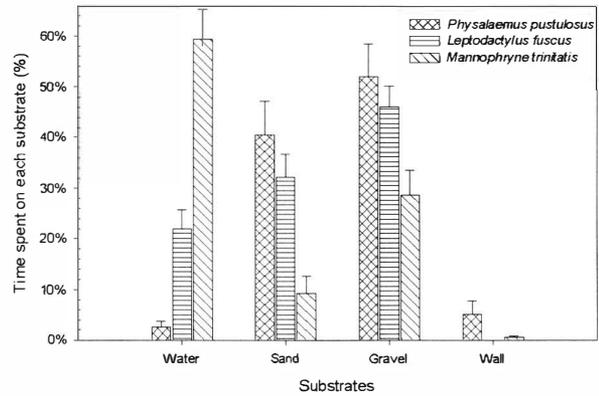


FIG 5. Mean time each species spent on each substrate in total, with SE. The results of ANOVA for each substrate are: water, $F_{2,27}=45.77, P<0.001$; sand, $F_{2,27}=9.37, P<0.005$; gravel, $F_{2,27}=7.83, P<0.005$; tank wall, $F_{2,27}=1.50, P>0.1$

restrial substrates, spending most time on gravel, which was significantly preferred to water or the tank wall. *L. fuscus*, which spent no time on the tank wall (factor omitted from analysis), showed a preference for gravel followed by sand then water. Water was significantly preferred by *M. trinitatis* to the other three remaining substrates, followed by gravel, sand and the tank wall.

The three species had very distinct preferences for the available substrates, with a significant difference in the amount of time spent by the three species on water, sand and gravel, but not the tank wall (Fig. 5). *M. trinitatis* spent the most time in water (almost 60%) compared to about 20% in *L. fuscus* and less than 5% in *P. pustulosus*.

ACTIVITY LEVELS

All three species remained inactive the majority of the time, with *P. pustulosus* being least active (Fig. 6) and *L. fuscus* (Fig. 7) and *M. trinitatis* (Fig. 8) remaining inactive for approximately 98% of the time. In *M. trinitatis*, jumping was a more prevalent activity compared to the other species, with a comparable amount of time spent jumping and turning. No frog of any species, was observed to feed during the timed observation peri-

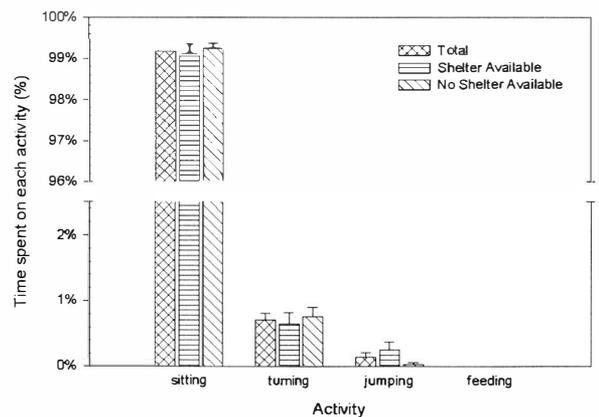


FIG 6. Mean time *P. pustulosus* spent engaged in each behaviour in total, and under both shelter treatments +SE. A break was inserted to allow the very small percentages of turning and jumping to be shown.

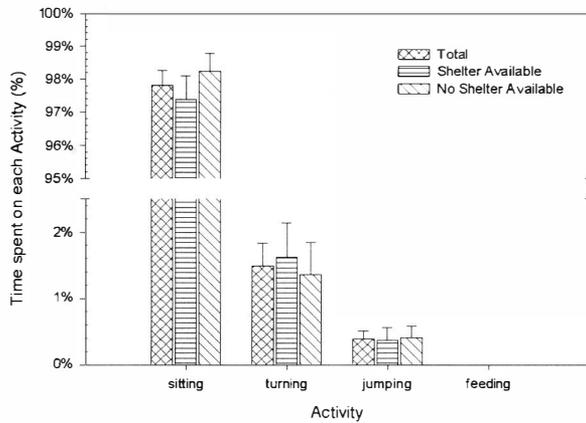


FIG. 7. Mean time *L. fuscus* spent engaged in each behaviour in total and under both shelter treatments \pm SE. A break was inserted to allow the very small percentages of turning and jumping to be shown.

ods, but feeding was witnessed at other times in both *P. pustulosus* and *M. trinitatis*. Although each species was predominantly inactive, there were differences in how activity was influence by disturbance and shelter treatment.

In *P. pustulosus*, individuals were observed to jump more frequently when shelters were available, but there was no difference in the level of other activities between the different shelter treatments (Table 4). Disturbance had no influence on activity levels in *P. pustulosus*. Conversely, *L. fuscus* exhibited no difference in activity levels between the shelter treatment groups, but when individuals were disturbed they showed an increase in activity (Table 4). There was no significant difference in the activity levels of *M. trinitatis* between the two

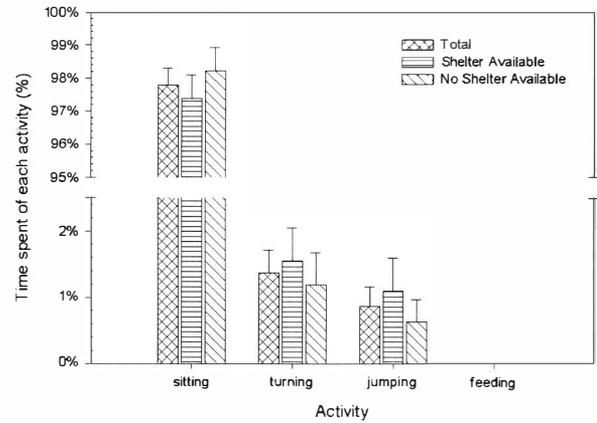


FIG. 8. Mean time *M. trinitatis* spent engaged in each behaviour in total and under both shelter treatments \pm SE. A break was inserted to allow the very small percentages of turning and jumping to be shown.

treatment groups ($\chi^2=1.62$, $df=2$, $P>0.05$) or between the activity levels when disturbed and undisturbed (Table 5, ($\chi^2=5.68$, $df=2$, $P>0.05$).

HUMIDITY

The mean relative humidity under the shelters for all substrates was $91.8\pm 0.5\%$ (mean \pm SE) while elsewhere in the tanks the relative humidity was lower at $86.8\pm 0.6\%$ (mean \pm SE). There was little difference in humidity, for each shelter condition, between the three substrates or between the two tanks. However, there was a significant difference between the relative humidity under the shelters and elsewhere in the tank ($U=152.0$, $P<0.001$).

TABLE 4. Results from multivariate analysis of activity levels for *P. pustulosus* and *L. fuscus*.

	Treatment	Activity	df	F	P
<i>P. pustulosus</i>	Shelter	Sitting	1	0.458	0.508
		Turning	1	0.022	0.883
		Jumping	1	4.965	0.041
	Disturbance	Sitting	1	0.069	0.796
		Turning	1	0.002	0.965
		Jumping	1	2.546	0.130
	Shelter \times Disturbance	Sitting	1	0.052	0.822
		Turning	1	0.003	0.954
		Jumping	1	0.691	0.418
<i>L. fuscus</i>	Shelter	Sitting	1	0.427	0.523
		Turning	1	0.641	0.435
		Jumping	1	0.058	0.812
	Disturbance	Sitting	1	4.730	0.045
		Turning	1	1.690	0.212
		Jumping	1	5.618	0.031
	Shelter \times Disturbance	Sitting	1	0.507	0.487
		Turning	1	0.304	0.589
		Jumping	1	0.234	0.635

TABLE 5. Number of three-second intervals observed for each activity under disturbed and normal conditions in the shelter group for *M. trinitatis*, used in chi-squared analysis.

	Turning	Sitting	Jumping
Disturbed	39	1549	12
Not Disturbed	16	1582	2

DISCUSSION

MORTALITY

M. trinitatis had a high mortality rate compared to the other two species, even though the treatment of all species was the same. We suspect that stress was the cause of the high mortality rate in *M. trinitatis*, since the rate of mortality decreased when disturbance from measuring was removed.

GROWTH RATE

When comparing the rate of increase in mass and SVL between the two shelter treatment groups for each of the three species, there were four cases, representing each species, where frogs grew faster with shelters present. Although there was no significant difference in the rate of growth between the two treatments in the remaining two comparisons – length in *P. pustulosus* and mass in *L. fuscus* – there was no instance where frogs without shelter grew faster than those with shelter.

The differences in growth rate between shelter and non-shelter groups were not large, and may have been affected by two factors: substrate and food suitability. Where shelters were not provided, frogs were able to burrow into the substrate, providing a self-made shelter and thereby reducing the practical difference between our two designs. It is noticeable that the species with the most consistent difference in growth rates (*M. trinitatis*: differences significant for both mass and SVL) spent most of its time in the water rather than on either of the solid substrates, and therefore less often burrowing.

The food available for all species was initially *Drosophila*, but as the frogs grew they may not have been able to consume enough *Drosophila* to maintain growth, and may even have ceased to forage when only *Drosophila* were available. We saw an effect of this sort in two of our species: in *P. pustulosus*, growth rate declined around week nine, with the experiment terminating soon after. In *L. fuscus*, growth on *Drosophila* plateaued after about five weeks, with the frogs seeming to lose interest in the food. Previous work which supports these observations, has shown that anurans change their diet as they grow and that larger individuals are more selective in the size of prey that they consume and may develop negative electivities for prey items that are no longer suitable (Lima, 1998; Newman, 1999). This highlights an important factor in captive rearing, that there may be a threshold age or size at which the diet of anurans changes, which could impact rearing programmes.

Growth resumed in *L. fuscus* when crickets were provided in week nine, and during the final growth phase

individuals grew faster in length and mass when shelters were provided. The diet of crickets may have allowed growth to resume, and at a higher rate for frogs with shelters, for two reasons: each cricket caught provides more food, for less effort, than a fruit fly; and crickets only move along the substrate, rather than being distributed three-dimensionally throughout the tanks, which may make foraging from shelters particularly efficient.

M. trinitatis did not show this effect since this species may continue to forage on small prey as it grows, for three possible reasons. First, adult *M. trinitatis* are the smallest of the three species and there is a relatively small ontogenetic size change from juvenile to adult. Second, the mountain stream habitat used by *M. trinitatis* may have sparse resources of larger prey. Thirdly, *M. trinitatis* may display species-specific foraging activities that do not change with size (Lima & Magnusson, 2000). Gut contents of adults taken from the field (Downie, unpublished observations) include prey items that are no larger than the *Drosophila* used in this study.

It is a possible concern that in two out of the three species used in this study (*P. pustulosus* and *L. fuscus*) the mean initial sizes of frogs in the shelter and non-shelter groups were significantly different. The initial size of frogs varied over a considerable range due to a one to four week post-metamorphic growth period prior to the start of the experiment, not because of inherent differential growth rates between individuals selected for the two treatment groups. We do not believe that these initial differences affected our conclusions because frogs with shelters provided grew faster than those without shelters for all species, whereas the differences in initial size were in the opposite direction in *L. fuscus* and *P. pustulosus*, and there was no such difference in *M. trinitatis*.

BENEFITS OF SHELTER

The obvious benefits of shelter to a juvenile amphibian are in foraging, where shelter may increase the success rate of an ambush predator; and in predator avoidance, where the availability of shelter provides a protected environment during quiescent periods and a convenient place to escape when threatened during active periods. In other taxa (squirrels: Dill & Houtman, 1989; fish: Dill, 1990; McLean & Godin, 1989) individuals have been shown to delay fleeing from threats the nearer they are to refuges, allowing increased foraging times and a reduction in energy expenditure on flight. In fish, Krause *et al.* (1998) have demonstrated that the potential foraging advantages provided by shelter are influenced by body size. Therefore there may be differences in the effects of shelter on growth rate between juvenile and adult individuals.

Less obviously, shelters may have thermoregulatory and osmoregulatory benefits, by providing suitable microclimates. Several studies have demonstrated that shelters can reduce physiological stresses caused by heat, cold weather and drought in terrestrial anurans

(Hoffman & Katz, 1989; Schwarzkopf & Alford, 1996; Parris, 1998; Seebacher & Alford, 2002). Although these factors might seem to be more a threat to survival than to growth, Lillywhite *et al.* (1973) have shown that in addition to metabolic costs involved in restoring water balance after desiccation there are physiological consequences, such as reduced oxygen consumption and metabolic capacity, that can reduce growth rate. In our study, we were able to detect a relative humidity advantage under the shelters, which may have contributed to the growth rate difference. Such benefits might be particularly important in juvenile frogs due to their relatively high surface area/volume ratios.

The aim of this study was to determine whether there was a detectable benefit to growth rate in shelter, rather than to identify the precise nature of the benefits. These could be investigated using different interspecies comparisons: for example, highly toxic species, like some bufonids and dendrobatids may be less influenced by predator avoidance benefits; highly terrestrial species like bufonids might be less reliant on physiological benefits.

Our comparison between shelter and non-shelter groups was complicated by the burrowing behaviour of both *L. fuscus* and *P. pustulosus*. However, Parris's (1998) study on juvenile *Rana* found that individuals that actively dug their own burrows conserved water less efficiently than those that used pre-existing shelters. Therefore, in addition to the energetic cost of active burrowing, there may be an osmotic cost which helps explain growth differences between shelter and non-shelter groups in the two burrowing species. The substrate we provided was rather shallow (about 1 cm) and it would be interesting to determine whether substrate depth had an impact on burrowing behaviour and growth.

SUBSTRATE CHOICE

In each species there were clear but distinct preferences for substrate type: *P. pustulosus* showed the most terrestrial preference, with hardly any time spent in water, and gravel and sand being fairly evenly utilized; *M. trinitatis* was the most aquatic of the three, with most time spent in water, and gravel preferred to sand; *L. fuscus* was intermediate between the other two, but gravel was preferred. These differences reflect well what is known of the natural history of these species. *P. pustulosus* is a toad-like anuran, which spends the majority of its time in terrestrial habitats, returning to water only for reproduction (Marsh *et al.*, 2000); *L. fuscus* is more frog-like, spending its time in damp burrows close to sources of water; *M. trinitatis*, the stream frog, lives permanently close to running streams in the rainforest.

Gravel was the preferred terrestrial substrate in all three species, although this trend was not always significant. Seebacher & Alford (2002) found a substrate preference for rocks over other substrates in *Bufo marinus*, although they did not provide sand as an alternative. Their results indicated that an uneven damp,

rocky surface helped reduce water loss, which could explain why all three species demonstrated a preference for gravel over sand.

In pilot studies on *L. fuscus* and *M. trinitatis* (Downie, unpublished observations), having the food source in a fixed location resulted in preferences that were correlated with the location of the *Drosophila* tubes. This suggests that when a food source is randomly encountered each species will show substrate preferences that reflect their natural habitat, but when food placement is fixed it heavily influences where individuals spend their time. Preliminary work on amphibian habitat enrichment has revealed that feed placement and treatment influences the duration and intensity of foraging behaviour and that amphibians are fully capable of 'learning' the feed-site (Campbell-Palmer, personal communication).

ACTIVITY LEVELS

Measured activity levels were low in all three species, but this is not surprising. *L. fuscus* and *P. pustulosus* are thought to be primarily nocturnal, and our observations were made during the day. However, *M. trinitatis* is diurnally active but showed only marginally higher activity levels than the other two species. In addition, we assessed activity before feeding (in order not to have the data dominated by foraging activity) and frogs were therefore observed at resting activity levels. Although there were differences in activity levels between the shelter groups they were mainly non-significant, so it is unlikely that activity contributed greatly to the differences in growth rates between treatments. Disturbance had an impact on activity level only in *L. fuscus* with a difference between shelter and non-shelter groups. The non-shelter group remained immobile, mainly in their burrows, when disturbed; many of the shelter group were out in the open, and disturbance led them to retreat rapidly under a shelter. For all species there was a similar finding whenever we were attempting to catch the frogs in order to measure them: those with access to shelters immediately jumped under them (personal observations).

IMPLICATIONS FOR CAPTIVE BREEDING PROGRAMMES

None of the species investigated here is currently endangered. However, there are implications in our results for captive management programmes. The provision of shelter was a positive feature in the growth rate of all three species, and should perhaps be universal in the rearing of juvenile frogs.

Handling seemed not to be a problem for two of the species, but seemed to be highly stressful for *M. trinitatis*. Knowledge of the impact of stress should be important in determining how to monitor growth.

The three species had different substrate preferences in the simple three-way choice we offered. Therefore, if knowledge of an endangered species' natural history is limited, then it should be possible to ascertain habitat preferences in many species by means of choice experi-

ments of this kind. Establishing the ideal habitat is likely to be important in achieving fast growth rates in juvenile amphibians and can be used to assess suitable locations for reintroduction or translocation sites.

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DIVERSE TYPES OF ADVERTISEMENT CALLS IN THE FROGS *EUPSOPHUS CALCARATUS* AND *E. ROSEUS* (LEPTODACTYLIDAE): A QUANTITATIVE COMPARISON

RAFAEL MÁRQUEZ¹, MARIO PENNA², PAULO MARQUES^{1,3} AND JOSÉ PEDRO S. DO AMARAL⁴

¹Fonoteca Zoológica, Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain

²Programa de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Santiago de Chile, Chile

³Museu Bocage, Museu Nacional de História Natural, Centro de Biologia Ambiental, Lisboa, Portugal

⁴Centro de Biologia Ambiental, Faculdade de Ciências da Universidade de Lisboa, Lisboa, Portugal

The variability of the advertisement calls of males from two Chilean populations of the leptodactylid frogs, *Eupsophus calcaratus* and *E. roseus* was studied and their calling behaviour further defined. Characteristic audio spectrograms and oscillograms for each species are presented. The spectral and temporal features of the calls were analysed, and intra-population and inter-specific differences in sound parameters were tested using correlation and discriminant function analysis. The calls of both species were tonal, had specific frequency modulation patterns (FM), and showed substantial inter-individual variation in several of their components. At least four discernible types of FM patterns were found in both species. Audio-spectrogram correlation and discriminant analysis showed that the quantitative characteristics of the calls of the species were clearly distinct; the most discriminating parameters were frequency, inter-call interval, and fundamental frequency.

Key words: behaviour, frequency modulation, leptodactylid, sound repertoire

INTRODUCTION

Although acoustic communication is an important feature of anuran social behaviour, each species generally has a limited repertoire of specific acoustic signals (Hauser, 1996; Bradbury & Vehrencamp, 1998). However, recent studies have revealed an important degree of plasticity in the calls of some anurans. For example in the spectral domain, male frogs may alter the dominant frequency of their calls in response to different features of acoustic stimuli (e.g., Lopez *et al.*, 1988; Wagner, 1989; Bee *et al.*, 2000; Given, 1999). In particular, an unprecedented complexity in frequency modulation (FM) has been recently reported for an Asian frog (Feng *et al.*, 2002).

The emission by males of a species-specific advertisement call and the selectivity of females for the call characteristics constitute the major pre-mating reproductive isolating mechanism in anurans (e.g. Asquith *et al.*, 1988; Gerhardt, 1974; Blair, 1958). Consequently, the study of advertisement calls is a tool that has been used extensively to elucidate taxonomic problems. Moreover, studies of mating call variation have provided useful tools for the development of evolutionary models, both at the inter-specific and inter-population level (e.g. Castellano *et al.*, 2002), and at the intra-population level in studies of communication, behaviour, and sexual selection (e.g., Friedl & Klump, 2002).

We determined the individual and populational variation of the advertisement calls of two species of leptodactylid frogs from Southern Chile: *Eupsophus calcaratus* and *Eupsophus roseus*. The advertisement calls of these two species, which have similar adult body sizes and adjacent distribution ranges, have been already described in the literature (*E. calcaratus*, Formas, 1985; *E. roseus*, Formas & Vera, 1980). However, these descriptions were based on a limited number of individuals and calls: three males and 15 calls of *E. calcaratus* (Formas, 1985) and three males and an undetermined number of calls of *E. roseus* (Formas & Vera, 1980). Although these descriptions did not provide sufficient information for the quantitative comparison of the calls of *E. calcaratus* and *E. roseus*, they hinted at the complex spectral structure of the calls. Therefore, we extended those preliminary descriptions of the calls (Formas, 1985; Formas & Vera, 1980) by analysing the variation of call parameters in two geographically disjunct populations of *E. calcaratus*, and *E. roseus*. We wanted to characterise the complex FM patterns of the calls of these frogs for ancillary studies on signal recognition and speciation processes.

MATERIAL AND METHODS

Recordings of the calls of *Eupsophus calcaratus* and *E. roseus* were obtained in Chile. Recordings of *E. calcaratus* were conducted in December 1995 and October 1996 and 1997 at La Picada, Parque Nacional Vicente Pérez Rosales, Osorno Province, X región (41°6' S, 72°30' W), and recordings of *E. roseus* were

Correspondence: R. Márquez, Fonoteca Zoológica, Departamento de Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain.
E-mail: rmarquez@mncn.csic.es

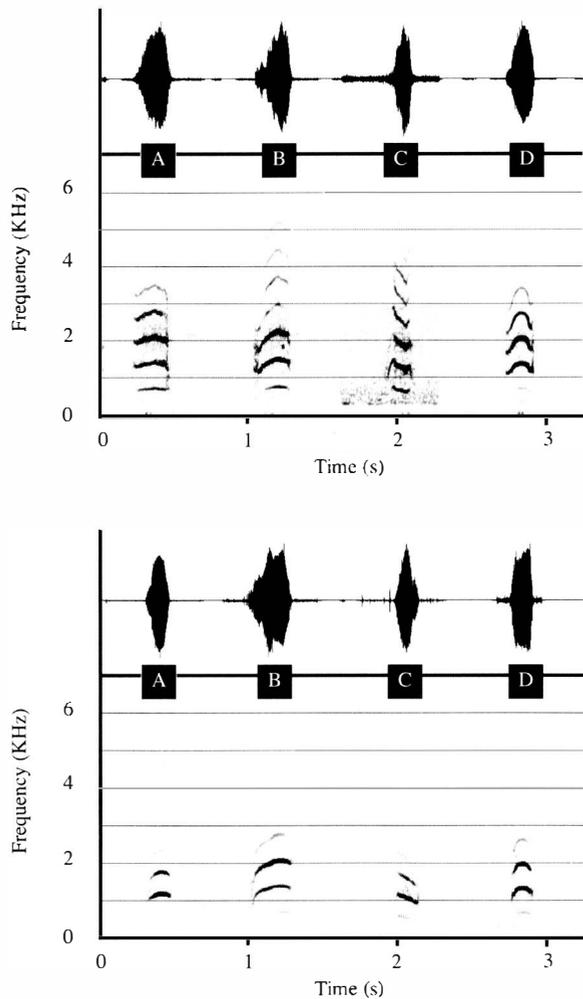


FIG. 1. Oscillogram and audio-spectrogram of the advertisement calls of four males of *Eupsophus calcaratus* and *E. roseus* with different patterns of frequency modulation (FM): A, almost flat FM; B, upwards FM; C, downwards FM; D, up-down FM. Fast Fourier Transform (FFT) details: frame width 1024 points, filter bandwidth 174 Hz, and overlap 75%.

for *E. roseus*. The advertisement calls of both species consisted of a single short FM note with a complex harmonic structure. The resulting sound resembled a short cat-like meow.

We made 30 recordings from each population. Of these recordings, a total of 263 calls from 24 individuals of *E. calcaratus* and 277 calls from 18 individuals of *E. roseus* were analysed. Some recordings included calls from more than one individual, either because they shared a portion of the burrow or because the openings

of the burrows were adjacent. These recordings were excluded from the numerical analysis. However, a single call from each of these recordings was used for audio-spectrogram correlation. Table 1 summarises the quantitative parameters measured. For some calls not all sound parameters could be determined because of interference of neighbouring callers or abiotic noise. Therefore, the number of recordings for which different parameters were analysed was not the same.

In both species, each male emitted a single FM pattern within a recording session. In the calls of *E. calcaratus*, at least four basic FM patterns were clearly discernible: almost flat FM (type A, 2 of 23 males), upward FM (type B, 1 of 23 males), downward FM (type C, 4 of 23 males), and up-down (inverted U) FM (type D, 16 of 23 males); there may be additional call types with characteristics intermediate to those described here. In a given night, the call FM pattern of an individual male was largely invariable during the recording session. Advertisement call duration varied from 112–262 ms. The advertisement calls of *E. calcaratus* (Fig 1A) had a harmonic structure; the spectral energy of the first harmonic (or the fundamental) was weak. The second or the third harmonics were dominant: the second harmonic varied from 1170–1486 Hz and the third harmonic ranged from 1817–2157 Hz. In *E. calcaratus*, RSA was consistently positive in the calls of 9 males (39.13%) and the dominant harmonic was the second. Eight males (34.78%) had negative values of RSA and a dominant third harmonic, and 6 males (26.09%) had calls with positive and negative values of RSA. This value could not be measured in one individual because of excessive background noise.

In the calls of *E. roseus* (Fig. 1B), the pattern in FM was consistent within recording and at least the same four different patterns as for *E. calcaratus* were observed: almost flat FM (type A, 2 of 17 males), upward FM (type B, 1 of 17 males), downward FM (type C, 4 of 17 males), and up-down (inverted U) FM (type D, 10 of 17 males), which was again the most common case. Advertisement call duration varied from 124–235 ms. Similar to *E. calcaratus*, calls from this species had two dominant harmonics, the second (range 1037–1285 Hz) or third (1618–1871 Hz) harmonics, with the fundamental having little or no energy. Similarly to what was measured in *E. calcaratus*, in some calls of *E. roseus* the emphasised frequency was the second harmonic, whereas in other individuals it was the third. In *E.*

TABLE 2. Statistics for the sound parameters of *Eupsophus calcaratus* and *E. roseus* that were significantly different. The suffixes 2 and 3 mean second and third harmonics, respectively. Hf high frequency of the harmonic, Lf low frequency of the harmonic, ICInt the inter-call interval, and Dur the duration of a call. R^2_{adj} = adjusted coefficient of determination; $t_{a,b}$ = Student's t value, a is sample size of *Eupsophus calcaratus* and b is sample size of *E. roseus*.

Hf2	Lf2	Hf3	Lf3	ICInt	Dur
$R^2_{adj}=0.44$	$R^2_{adj}=0.26$	$R^2_{adj}=0.60$	$R^2_{adj}=0.27$	$R^2_{adj}=0.07$	$R^2_{adj}=0.16$
$t_{23,17}=5.67$	$t_{23,17}=3.84$	$t_{23,18}=7.74$	$t_{23,18}=4.03$	$t_{23,18}=2.24$	$t_{24,18}=3.20$
$P<0.0001$	$P=0.0005$	$P<0.0001$	$P=0.0003$	$P=0.0493$	$P<0.0001$

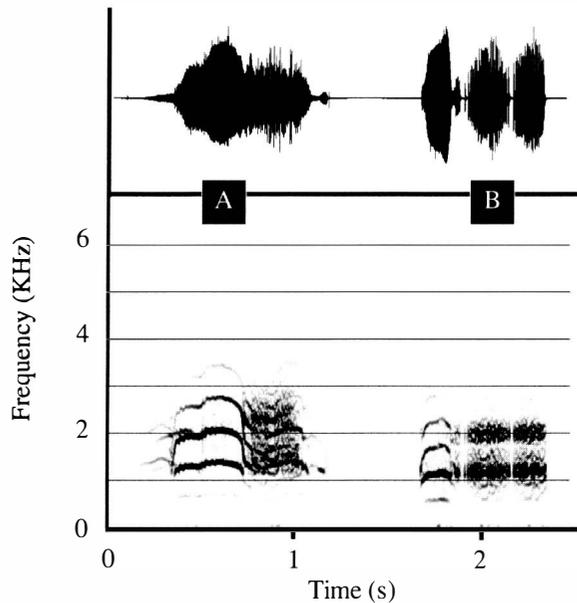


FIG. 2. Oscillogram and audiospectrogram of the aggressive calls of *Eupsophus calcaratus* (A) and *E. roseus* (B). Fast Fourier Transform (FFT) details: frame width 1024 points, filter bandwidth 174 Hz, and overlap 75%.

roseus, RSA was consistently positive in six males (35.29%), five males (29.41%) had consistently negative values, and six males (35.29%) showed positive and negative values. This value could not be measured in one individual because of excessive background noise.

Considering the four types of call-FM pattern, the pooled within-type mean correlations were significantly higher than the pooled among-type mean correlations for both species of *Eupsophus* that we studied (*E. calcaratus* $R^2_{adj}=0.01, T=-2.24, P=0.03$; *E. roseus*

TABLE 3. Means (r) and standard deviations (s) of the audio-spectrogram correlations between and within populations of *Eupsophus calcaratus* and *E. roseus*. Self-correlations were excluded in the within population samples. * $P<0.01$.

	No.	r	s
<i>E. calcaratus</i> vs. <i>E. roseus</i>	900	0.5021*	0.08979
<i>E. calcaratus</i> vs. <i>E. calcaratus</i>	435	0.5788	0.07663
<i>E. roseus</i> vs. <i>E. roseus</i>	435	0.5675	0.10415

TABLE 4. Summary statistics of discriminant functions and correlations between original variables and discriminant functions. * show large absolute correlations between each variable and any discriminant function. Wilks' lambda = 0.03327; Wilks' lambda exact $F_{3,33}=22.0589, P<0.0001$.

STATISTIC		Discriminant Functions	
		Canonical 1	Canonical 2
	Eigen value	2.01	-1.11×10^{-16}
	Percent	100	0
	Canonical correlation	0.8169	0
SCORING COEFFICIENTS	Hf3	0.0113*	-0.0027
	IntCalD	0.1385	0.2941*
	Fundf	0.0015*	-0.0004

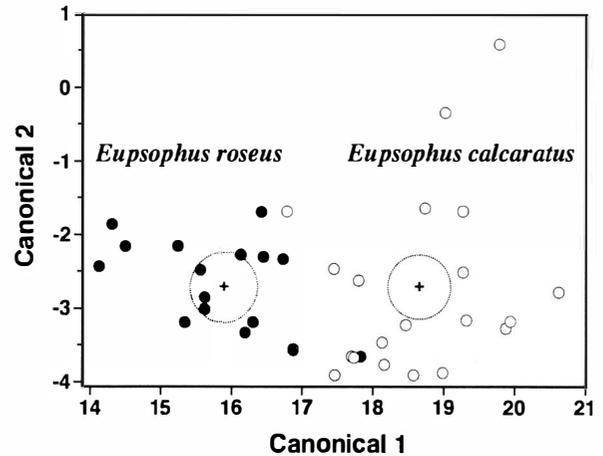


FIG. 3. Canonical functions 1 and 2 from discriminant functions analysis (DFA) performed on three sound parameters: the high-frequency of the third harmonic (Hf3), the inter-call interval (IntCalD), and the fundamental frequency (Fundf), for *Eupsophus calcaratus* and *E. roseus*. The size of the dotted circle corresponds to a 95% confidence limit for the mean.

$R^2_{adj}=0.07, T=-5.64, P<0.0001$). Furthermore, the statistical comparison of individual call parameters between species showed that the frequencies of the emphasised harmonics (second and third) were all significantly different between species (Table 2), and that the duration and inter-call interval were also significantly different (Table 2). However, fundamental frequency, rise time proportion, and RSA were not significantly different between species.

In addition to the advertisement calls, a limited number of aggressive calls was recorded in both populations (three aggressive calls of one individual for *E. calcaratus*, and one aggressive call for *E. roseus*). We considered these calls to be aggressive because they were often emitted at the end of a vigorous exchange of advertisement calls among neighbouring males, or when stimulated by a loud whistle from the researchers. The oscillograms and audiospectrograms of the aggressive calls are shown in Fig 2. In the field, we observed that the aggressive calls of *E. calcaratus* promptly triggered

a neighbouring sympatric male (*Eupsophus emiliopugini*) to emit his advertisement calls. However, the number of recordings of aggressive calls is not sufficient for us to elaborate about comparative aspects.

The comparison of the calls from the two species through audio-spectrogram correlation yielded the results shown in Table 3. Given that a single call was used per male in the audio-spectrogram batch correlation, this test allowed us to use calls from all the recordings obtained (30 from each population). The average correlation was significantly higher in both within-species audio-spectrogram correlations than in the between-species audio-spectrogram correlations (*E. calcaratus* $t=15.32$, $df=1333$, $P<0.0001$). Furthermore, the average within-species correlation of *E. calcaratus* was not significantly higher than that of *E. roseus* ($t=1.82$, $df=868$, $P=0.0687$). The variation in call characteristics was greater between-species than within-species, and the within-species sample from *E. calcaratus* was slightly more homogeneous than that of *E. roseus*.

The two frog species could be discriminated by DFA based on the sound properties of their calls (Fig. 3, Table 4). As determined by stepwise DFA, the sound parameter that best discriminated the two species was Hf3, followed by ICInt, and finally Fundf. Only two individuals were incorrectly classified by the test (one of each species).

DISCUSSION

The presence of four basic FM patterns in advertisement calls of *E. calcaratus* and *E. roseus* is remarkable, particularly the predominance of upward and downward FM sweep calls (type D calls). These taxa have been studied extensively from a genetic standpoint (Formas *et al.*, 1991; Formas, 1985; Formas & Brieva, 1992; Formas *et al.*, 1992; Nuñez *et al.*, 1999). It is unlikely that more than one species would be found in a single location. Therefore, the shared complex FM patterns between the species suggests a relatively recent common ancestor.

Possibly, the presence of different types of calls in these frogs is related to the relaxation of the selective pressures for call stability usually imposed by the characteristics of the acoustic environmental of anurans. In Southern Chile, the sound environment where these frogs communicate is relatively simple, and these species have few or no acoustical competitors (Penna & Veloso, 1990). *Eupsophus calcaratus* and *E. roseus* call in late winter and spring, while most other anuran species are not active. At La Picada, the only species with a calling activity period that may overlap with the calling activity of *E. calcaratus* was the congeneric species *E. emiliopugini*, and that overlap might happen only in late spring. However, the calls of *E. emiliopugini* have a substantially lower frequency (Penna & Solís, 1999; Penna & Solís, 1996). Furthermore, during this study no arthropods or nocturnal birds called nearby the breeding aggregations of these frogs.

The wide amplitude modulation range of the calls of these species of *Eupsophus* (above 1 kHz in some cases) may be related to the resonance characteristics of the burrow-like cavities in the moss from which males call (Penna, unpublished data). Given that burrows are likely to vary in shape and size, this variability in burrow filtering may also explain why small-scale features of the call may not be salient. Each cavity amplifies a specific frequency, and a considerable variation in resonant frequency occurs among cavities (842-1836 Hz for *E. calcaratus*, Penna, unpublished data). In *E. emiliopugini*, which is also fossorial and calls from burrow-like cavities, the reception of amplified conspecific calls by males inside cavities may influence the vocal interactions in choruses by amplifying calls with lower frequencies (Penna & Solís, 1996), and similar processes may also happen in the two species of *Eupsophus* that we studied. Moreover, if female frogs are in underground cavities when they first hear the advertisement calls of males, then female frogs initially will hear and potentially be attracted to male sounds amplified by both the caller's and their own cavities, and later will orient to male calls that are exclusively amplified by the caller's cavity. However, virtually nothing is known about these issues of the biology of females.

A high diversity in FM patterns has been recently reported for an Asian ranid frog, *Amolops tormotus* (Feng *et al.*, 2002). So rich is this diversity of FM patterns that no two calls were the same in over 12 hours of recordings from 21 individuals (Feng *et al.*, 2002). Although with a relatively diverse sound repertoire, the two species of *Eupsophus* that we studied are not as richly diverse as *A. tormotus*. Moreover, individuals of *A. tormotus* have a large sound repertoire, whereas the individuals of the two species of *Eupsophus* that we studied seem to emit only one call out of their species' repertoire.

Formas (1985) described the characteristics of the advertisement call of *E. calcaratus* from Puntra (Chiloé Province, Chile) and reported only one type of call with an upward and downward FM sweep, which corresponds to our type D call. The call durations measured in that description (0.15-0.21 s) are similar to the values obtained by us, whereas the reported dominant frequency range (2.2-3.8 kHz) is clearly above the frequencies measured in our study.

Formas & Vera (1980) also reported only one type of call (our type D) based on calls from three individuals of *E. roseus* from Huachocopihue, near the city of Valdivia, Chile. They reported slightly longer calls (0.19-0.2 s) and substantially higher dominant frequencies (1.6-2.9 kHz) than those measured by us in the Tinquilco population. In a more recent comparative study, an included audio-spectrogram of *E. roseus* also showed only type D calls (Formas & Brieva, 1992). Penna & Veloso (1990) also described vocalisation of *E. roseus* from Valdivia as type D calls. In relation to the harmonic structure, these authors also reported a long

call similar to the aggressive call that we recorded. Other call characteristics that they reported, e.g., the second harmonic was the dominant frequency, but also recognised upper harmonics as having considerable power. The dominant second harmonic, the note durations (70-160 ms), and the dominant frequencies (0.7-1.1 kHz) are in agreement with the present observation.

In the present study, the comparative analysis of the calls of *E. calcaratus* and *E. roseus* showed an important structural similitude between the two species. The signals had at least two dominant harmonics and showed a pronounced FM in most individuals. In some cases, the range of frequency excursion was above 1 kHz. However, both the audio-spectrogram correlations and the discriminant test showed differences between the calls of the two species.

In both species, male advertisement calls showed remarkable inter-individual variation and had within individual, within-recording homogeneity. Because we recorded frogs during relatively short periods and given that most recordings were obtained from individuals that were not captured, we could not ascertain whether a male could emit more than one different call from these species' repertoires during more extended periods. However, during extended recordings of eight males of *E. calcaratus* that lasted 22-77 minutes, the individuals did not change their call type over the time sampled (Penna, unpublished data).

A further step in the study of these species could be to determine whether these frogs can discriminate among their call types, and additionally to determine which physical characteristics of the males determine the type of call produced. Furthermore, it would be interesting to determine how females perceive and respond to a given call amplified by both caller and female cavities, or exclusively by the caller's cavity. Eventually – and as has been done for *Physalaemus pustulosus* (Wilczynski et al., 2001) – one could determine the extent to which the preexisting features of the receiver systems of *Eupsophus* have conditioned the evolution of their calls.

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EGG MORTALITY AND EARLY EMBRYO HATCHING CAUSED BY FUNGAL INFECTION OF IBERIAN ROCK LIZARD (*LACERTA MONTICOLA*) CLUTCHES

PEDRO LOPES MOREIRA¹ AND MARGARIDA BARATA²

¹Centro de Biologia Ambiental and ²Centro de Micologia, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, Portugal

Infertile and non-viable fertile eggs within a reptile clutch may decrease the incubation success of the remaining eggs, as (1) opportunistic pathogens may use the nutrient resources provided by dead eggs to colonize the clutch and spread to and kill viable eggs; and (2) odours released by spoilt eggs may attract predators to the clutch. These hypotheses were tested on the Iberian rock lizard (*Lacerta monticola*) by comparing the incubation success of fertile eggs between clutches composed solely of fertile eggs and clutches containing a small number of dead eggs. In a laboratory experiment, fungi (*Fusarium* sp. and *Gliocladium* sp.) colonized both infertile eggs and fertile eggs that died during incubation and thereafter spread to and killed adjacent eggs. In addition, offspring hatched earlier from fungal infected eggs than from non-infected eggs. The former were smaller and lighter than the latter, as they hatched before using the full egg yolk content. Results from a field experiment did not corroborate the fungal pathogenic effects observed in the laboratory nor did they confirm that clutches containing dead eggs suffer higher predation. Despite the inconclusive results regarding the role of fungal pathogens in nature, the present study indicates that this subject deserves further investigation in reptiles.

Key words: egg infertility, egg viability, fungal pathogen, early hatching

INTRODUCTION

Many oviparous female reptiles deposit a clutch comprised of several eggs in a nest, with eggs in close proximity or even adhered to each other, without providing care to eggs or hatchlings (Greene, 1997; Pianka & Vitt, 2003). They may provide good model systems to investigate whether infertile and non-viable fertile eggs within a clutch decrease the hatching success of the remaining eggs or the offspring quality. These effects are little studied in reptiles, but they may exert important selective pressures on reproductive traits. Females that lay a single (or only a few) infertile or non-viable fertile eggs within a clutch may jeopardize their entire reproductive output if dead eggs promote clutch colonization by pathogens that kill eggs (Smith *et al.*, 1985; Green, 1999; Robinson *et al.*, 2003), trigger early hatching of embryos (Warkentin *et al.*, 2001; Wedekind, 2002), or infect the tissues of hatchlings (J. Wyneken, unpubl. obs., cited in Eckert & Eckert, 1990). In addition, dead eggs may increase the risk of clutch predation by species attracted by the odor of spoilt eggs (Groves, 1982; Somma, 1989).

Saprolegniaceae water moulds are frequent opportunistic pathogens of fish and amphibian eggs since zoospores readily colonize dead eggs and hyphae thereafter spread to and kill adjacent viable eggs (Smith *et al.*, 1985; Green, 1999; Robinson *et al.*, 2003). Consequently, amphibian communal spawners may suffer from higher egg mortality caused by fungi than species that

space their eggs out (Blaustein *et al.*, 1994; Kiesecker & Blaustein, 1997). Low temperature and pH (Beattie *et al.*, 1991; Bellemakers & Van Dam, 1992), as well as high levels of ultraviolet-B radiation (Kiesecker & Blaustein, 1995), can contribute to egg mortality as they decrease embryo resistance to fungal pathogens. In terrestrial environments, fungal infection of amphibian clutches can also start on dead eggs (Tilley, 1972; Forester, 1979). Females that brood the clutch can reduce egg mortality, as oophagy of dead/infected eggs prevents fungi from spreading to viable eggs and egg mechanical agitation retards fungal growth by disrupting the mycelia (Tilley, 1972; Forester, 1979). As such, egg-laying behaviour and parental care in amphibians have been suggested to have evolved as a defence against egg pathogens (reviewed by Green, 1999). Moreover, embryos themselves may have evolved strategies to escape from pathogens, as both fish (Wedekind, 2002) and amphibian (Warkentin *et al.*, 2001) embryos may hatch earlier from pathogen-infected than from non-infected eggs.

Fungi have also been recognized to contribute to the mortality of lizard, snake and sea turtle eggs (Fitch & Fitch, 1968; Tracy, 1980; Phillott & Parmeter, 2001b). The first appearance of fungi (*Fusarium solani* and *Pseudallescheria boydii*) in sea turtle nests was always on a non-viable egg, and fungal hyphae from this failed egg then spread to and killed adjacent eggs (Phillott & Parmeter, 2001b). Indirect evidence that fungi can be pathogenic to reptilian eggs is provided by the observation that oophagy and removal of dead/infected eggs from the clutch occurs in several lizards with parental behaviour (Mitchell & Groves, 1993; Somma, 2003). These behaviours were hypothesized to prevent both

Correspondence: P. L. Moreira, Centro de Biologia Ambiental, Faculdade de Ciências da Universidade de Lisboa, Edifício C2, Campo Grande, 1749-016 Lisboa, Portugal. *E-mail:* plmoreira@netcabo.pt

the spread of pathogens to viable eggs and the detection of the female and/or clutch by predators attracted by the odor of spoilt eggs (Groves, 1982; Somma, 1989).

The present study used the Iberian rock lizard (*Lacerta monticola*) to address the hypotheses that (1) opportunistic pathogens use the nutrient resources provided by dead eggs to colonize the clutch and spread to and kill viable eggs, and that (2) odours released by spoilt eggs attract predators to the clutch. The incubation success of fertile eggs was compared between clutches composed solely of fertile eggs and clutches containing a small number of dead eggs (infertile eggs or fertile eggs killed by freezing), both in the laboratory and in the field. The experimental study was complemented with the identification of the fungi that infected clutches in the laboratory experiment and with the evaluation of the frequencies of egg infertility, egg fungal infection, and egg predation in nature.

MATERIAL AND METHODS

STUDY POPULATION

The Iberian rock lizard is a small insectivorous lacertid endemic to the Iberian Peninsula. In Portugal, it is restricted to a single population at the Serra da Estrela mountain. Lizards are active from March-May to October-November. Adult males emerge from winter hibernation 1-2 weeks prior to adult females, with the copulation season starting soon after female emergence and lasting for 2-4 weeks. Females copulate more than 4-8 times, frequently with several different males, and produce a single clutch per year with 2-11 eggs. Females deposit the entire clutch in shallow burrows that they excavate under rocks (pers. observation), 1-2 months after copulations (Moreira, 2002). They do not appear to provide any form of parental care to eggs or hatchlings (Somma, 2003; and pers. observation).

LABORATORY EXPERIMENT

In order to obtain both fertile and infertile eggs, Iberian rock lizards were bred in captivity and females were allowed to copulate a small number of times. Sixteen males and 44 females were caught on 11-15 October 1998, before entering winter hibernation, near Torre, the top of Serra da Estrela at 1993 m of altitude. Ten other females, caught on 26-30 March 1998 in the same area, were also used. The two groups of lizards were hibernated in captivity on 17 and 22 October 1998, respectively. Males were removed from hibernation on 8 February and females on 15 February 1999 (detailed rearing conditions in Moreira & Birkhead, 2003). Eighty-four copulations occurred between 22 February and 12 March 1999, involving 13 males and 43 females. Females were transferred to terraria without males once they had copulated the required number of times: 24 females copulated once, 11 copulated twice and eight copulated four or more times. When gravid, females were transferred to egg-laying chambers provided with

humid vermiculite as substrate. Eggs were collected soon after being laid. They were weighed (to the nearest 0.01 g) and candled to determine whether they were fertile or not (Olsson & Shine, 1997). Fertile eggs were numbered with a soft graphite pencil on the opposite side of the blastodisc. Forty females oviposited between 23 March and 14 April 1999 and provided 35 infertile and 184 fertile eggs.

Seventeen clutches consisting of six fertile eggs (hereafter termed fertile clutches) and 13 clutches consisting of four fertile plus two infertile eggs (hereafter termed mixed clutches) were assembled from eggs obtained from different females. A maximum of two fertile eggs per female was used per clutch in order to minimize family effects and each clutch was completed within a maximum of three days. Clutches were incubated separately in 600 ml plastic boxes (12 × 9 × 6 cm³) containing 400 ml of incubation medium (1 ml of demineralized water for 10 ml of vermiculite size '2'; Olsson & Shine, 1997) mixed with 20 ml of soil, collected on 21 March 1999 at the site selected for the field experiment (see below). Clutches were placed in the centre of the incubation boxes, and the eggs were arranged in two parallel rows of three eggs each, with eggs touching adjacent ones and their numbers facing up. The two infertile eggs in mixed clutches were placed in the outer positions of each row and both on the same end side of the clutch, either on the right- (six cases) or the left-hand side (seven cases; Fig. 1). Clutches were covered with about 5 mm of incubation medium. Incubation boxes were airtight closed, maintained in the incubator at 26°-28°C and opened regularly for air renewal.

Clutches were inspected at about the middle of the incubation period (16-24 days after the fertile eggs were laid; previous incubations under the same humidity and temperature conditions lasted 36-39 days) and fungal abundance was recorded according to three levels: (1) mycelia not visible to the naked eye; (2) mycelia covering part (less than half) of the clutch; (3) mycelia covering most (more than half) of the clutch. The eggs were then carefully uncovered, so that they were not displaced nor the mycelia disrupted, and categorized as being dead (collapsed), alive (turgid and with white eggshell), and infected by fungi (mycelia closely associated with their surface). Eggs were covered again after examination. Towards the end of incubation, clutches were inspected daily for hatched lizards, and fungal abundance and egg condition were recorded as described above. Forty-seven offspring hatched between 2-15 May 1999, and were measured (snout-vent length and tail length to the nearest millimeter, and head size to the nearest 0.05 mm) and weighed (to the nearest 0.01 g). Eggshells were examined for noticeable yolk residues. Adult lizards and hatchlings were released near Torre.

Eggs that died during incubation and eggshells from hatched eggs belonging to each of the clutches were preserved in 7 ml tubes filled to the top with 70% ethanol. Fungal spores and hyphae were abundant in the alcohol

solution, which allowed the microscopic identification (to the genus) of most fungi that infected the clutches. The fungi characteristic spores were counted on 30 field scopes (400× magnification) in each of two slide preparations. Clutches were considered to have been infected by a particular fungus when its characteristic spores were detected in the counting procedure.

FIELD EXPERIMENT

Eggs were obtained from 39 gravid females captured near Torre between 25 June and 6 July 1999. These females laid in egg-laying chambers (as above) and altogether produced one infertile and 235 fertile eggs. Seventeen other infertile eggs were obtained from three females that laid in captivity without copulating, which had been previously captured between 24 May and 16 June 1999 in the same area. In order to build the mixed clutches, and because infertile eggs were insufficient in number (only 18 available), 20 fertile eggs killed by freezing were also used. Eggs were sorted into 19 fertile clutches (six fertile eggs) and 19 mixed clutches (four fertile plus two dead eggs) in the same manner as for the laboratory experiment.

Fertile and mixed clutches were placed to incubate under flat rocks on 7-10 July 1999 near the Lagoa Comprida lagoon (1580 m of altitude), at a site where a large number of females oviposited every year. Each clutch was placed in the field within a maximum of eight days (mean±SD = 4±1.5 days) after its live eggs were laid. Flat rocks (c. 20-60 cm diameter × c. 5-10 cm thick) were prepared in advance (April 1999) and distributed in clearings over an area of about 50 × 30 m². The soil underneath the rocks was slightly ploughed so that the rocks were well sited on the ground and the soil humidity was preserved. Clutches were placed within a PVC plastic ring (11 cm diameter × 6 cm high; inserted in the ground beneath the centre of each rock) and were covered with about 10 mm of soil. Plastic rings prevented clutches from being crushed when rocks were laid over them and were perforated to allow for water exchange and access of potential predators to the clutch. Eggs in each clutch were spatially arranged as for the laboratory experiment (Fig. 1). Fertile and mixed clutches were equally distributed over the study area.

Clutches were first inspected on 20-22 July 1999 (12-13 days after the beginning of incubation in the field and up to 21 days after its live eggs were laid). Rocks were lifted and eggs uncovered by sweeping the soil with a brush. It was next recorded whether eggs were predated (eggs missing, being destroyed by ants, or with perforations similar to the latter) and also the condition of the remainder eggs (as for the laboratory experiment). After the inspection, the soil and rock were put back in place. Clutches were inspected again on 13-14 August 1999 (35-37 days after the beginning of incubation in the field). None of the eggs had hatched. The condition of the eggs was recorded (as above) and they were brought to the laboratory for the remainder of the incubation period. Females and offspring were released near Torre.

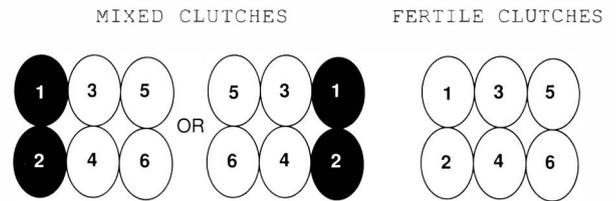


FIG. 1. Egg arrangement of infertile (black) and fertile (white) eggs in the mixed (4 fertile + 2 infertile eggs) and fertile (6 fertile eggs) clutches incubated in the laboratory. For the purpose of analyses, infertile eggs were in positions 1 and 2, fertile eggs that touched them were in positions 3 and 4, and fertile eggs that did not touch infertile eggs were in positions 5 and 6. For the field experiment, both infertile eggs and fertile eggs killed by freezing were used in positions 1 and 2 of the mixed clutches.

EGG INFERTILITY, FUNGAL INFECTION AND PREDATION IN NATURE

The frequency of egg infertility in the population was estimated from 103 clutches laid in captivity by females that were captured when gravid near Lagoa Comprida and near Torre between 1995 and 1999 (Moreira, 2002). The frequencies of egg fungal infection and egg predation in nature were estimated from unhatched eggs and eggshells that were dug out from lizard nests at a site near Torre where a large number of females oviposited every year. These nests were dug out on 28 August and 5 September 1998 (28 and 35 days, respectively, after the beginning of the egg-laying season in that year). A total of 132 unhatched eggs were collected and it was recorded whether they were predated and the condition of the remainder eggs (as for the field experiment). In addition, 348 eggshells from previous years were collected by sieving the soil removed to expose nests. Eggshells were classified as belonging to (1) hatched eggs (eggshells with a slit similar to that observed in eggs that hatched in captivity), (2) unhatched eggs (eggshells not opened), and (3) eggs predated by ants (eggshells with perforations similar to those observed in eggs being predated by ants). Sixty-two much degraded eggshells could not be ascertained to any of the above categories and were not considered for analyses. The unhatched eggs were taken to the laboratory for the remainder of incubation and offspring were released near Torre.

RESULTS

LABORATORY EXPERIMENT

Fungi readily colonized infertile eggs and grew to cover the majority of the mixed clutches. At the middle of the incubation period, all infertile eggs were closely surrounded by mycelia. At this stage, fungal abundance was higher in the mixed clutches (8% clutches with mycelium over part of the clutch, 92% clutches with mycelium over most of the clutch; $n=13$) than in the fertile clutches (35% clutches without visible mycelium, 35% clutches with mycelium over part of the clutch, 30% clutches with mycelium over most of the clutch; $n=17$; $\chi^2=2.1$, $df=2$, $P<0.01$). At the end of incubation, fungal abundance was higher in the mixed clutches

(100% clutches with mycelium over most of the clutch) than in the fertile clutches (18% clutches without visible mycelium, 12% clutches with mycelium over part of the clutch, 70% clutches with mycelium over most of the clutch), but differences were no longer statistically significant ($\chi^2=4.6$, $df=2$, $P>0.10$). This was because fungi also infected fertile clutches by colonizing the fertile eggs that died during incubation.

Fungal spores or hyphae were not detected in the three clutches that were not surrounded by visible mycelia. Spores of *Fusarium* sp. and *Gliocladium* sp. were the most prevalent and abundant among the 27 clutches that were surrounded by visible mycelia. Nineteen of these clutches (70.4%) were infected by *Fusarium* sp., one (3.7%) was infected by *Gliocladium* sp. and six (22.2%) were infected by both *Fusarium* sp. and *Gliocladium* sp. One clutch (3.7%) was infected by a fungus that could not be identified.

The hypothesis that opportunistic pathogens use the nutrient resources provided by dead eggs (infertile or non-viable fertile eggs) to colonize the clutch and spread to and kill viable eggs was supported by results of the laboratory experiment. Both at the middle and the end of incubation, the average proportion of fertile eggs that died per clutch was significantly higher in the mixed than in the fertile clutches (Fig. 2a). Two lines of evidence suggest that fungi were pathogenic rather than simply saprotrophic. Firstly, mycelia were observed in close association with the surface of eggs that appeared to be alive (turgid and with white eggshell), and even with eggs that eventually hatched. Secondly, the spatial

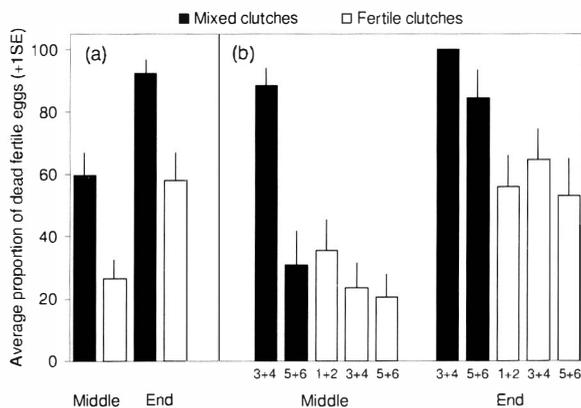


FIG. 2. (a) The average proportion of fertile eggs that died per clutch at the middle and the end of the laboratory experiment was higher in the mixed clutches (4 fertile + 2 infertile eggs; $n=13$) than in the fertile clutches (6 fertile eggs; $n=17$) (Mann-Whitney U -test: Middle: $z=3.33$, $P<0.001$. End: $z=2.97$, $P=0.003$). (b) The average proportion of fertile eggs that died per clutch in the mixed clutches was higher among the eggs (positions 3 and 4) that contacted with infertile eggs (positions 1 and 2) than among the eggs that did not (positions 5 and 6) (results were not statistically significant at the end of the incubation period) (Middle: $z=3.21$, $P=0.001$. End: $z=1.23$, $P=0.32$). In the fertile clutches, the average proportion of fertile eggs that died per clutch at the middle and the end of incubation did not differ significantly according to their position in the clutch (Middle: $z=0.64$, $P=0.52$. End: $z=1.00$, $P=0.22$).

pattern of egg mortality was consistent with a pathogenic basis. In the mixed clutches, the average proportion of fertile eggs that died per clutch was higher among the eggs that were in contact with infertile eggs than among the eggs that were not (results were not statistically significant at the end of incubation; Fig. 2b). Therefore, fungi that colonized infertile eggs (positions 1 and 2) first got in contact and killed adjacent fertile eggs (positions 3 and 4) and then spread from the latter to fertile eggs further away (positions 5 and 6). This pattern of egg mortality was not observed in the fertile clutches (Fig. 2b).

Non-viable fertile eggs also promoted fungal infection of fertile clutches. The pathogenic basis for egg mortality in fertile clutches was supported by the observation that the proportion of eggs that died between the middle and the end of incubation was higher among eggs that were in contact (laterally or diagonally) with eggs that were already dead at the middle of incubation (67%) than among the eggs that were in contact with eggs that were alive at that stage (33%; $\chi^2=4.2$, $df=1$, $P=0.04$). Nonetheless, non-viable fertile eggs were less costly to the remainder of the clutch than infertile eggs, as the proportion of fertile eggs that died in the 14 fertile clutches that were covered by mycelia (70%) was lower than in the 13 (all covered by mycelia) mixed clutches (92%; $\chi^2=9.3$, $df=1$, $P<0.01$).

Among the fertile and mixed clutches, 14 offspring hatched from eggs belonging to 12 different females that were closely surrounded by mycelia and 33 hatched from non-infected eggs belonging to 16 different females. The former hatched significantly earlier and were smaller in every respect and lighter than the latter (Table 1). Large yolk residues were only observed inside the shells of infected eggs, indicating that offspring hatched from these eggs before using the full egg yolk content. The ratio between the average offspring mass and the average original egg mass at oviposition was lower for the offspring that hatched from infected (0.82) than from non-infected eggs (1.00), despite the fact that the average original egg mass was higher for the offspring that hatched from infected eggs (Table 1).

FIELD EXPERIMENT

The hypotheses that dead eggs increase the risks of clutch predation or egg mortality by fungal pathogens were not supported by results of the field experiment. The incubation success of fertile eggs was not lower in the mixed clutches than in the fertile clutches, both when clutch predation and egg mortality not related to predation were analyzed separately and combined. Contrary to predictions, egg mortality tended to be lower among the mixed clutches.

When clutches were first inspected, one fertile clutch contained a broken dried egg (possibly damaged while putting the rock in place) and was therefore excluded from the analyses. At this stage, none of the eggs of the mixed clutches had been predated (0% predated

TABLE 1. Offspring that hatched from fungal infected eggs and from non-infected eggs during the laboratory incubation of mixed clutches (4 fertile + 2 infertile eggs) and fertile clutches (6 fertile eggs), and corresponding duration of incubation, snout-vent length (SVL), tail length, head size and body mass (mean \pm SD). The original egg mass at oviposition for both samples is also shown. Differences were statistically significant between the two groups in every respect (ANOVA). * one missing value for the tail length of a offspring that hatched from a non-infected egg.

	Infected	Non-infected	<i>F</i>	df	<i>P</i>
Offspring (number)	14	33			
Incubation (days)	34.1 \pm 0.86	37.2 \pm 1.04	91	1,45	<0.01
SVL (mm)	28.9 \pm 1.07	29.6 \pm 0.83	5.5	1,45	0.02
Tail length (mm)	36.8 \pm 4.64	41.0 \pm 2.44	17	1,44*	<0.01
Head size (mm)	7.05 \pm 0.23	7.25 \pm 0.21	8.1	1,45	0.01
Mass (g)	0.371 \pm 0.450	0.423 \pm 0.035	18	1,45	<0.01
Original egg mass (g)	0.455 \pm 0.033	0.424 \pm 0.043	5	1,45	0.04

clutches). Ants were destroying four eggs in one fertile clutch and two eggs were missing from another (11% predated clutches). At the second inspection, four fertile eggs were perforated in two mixed clutches (11% predated clutches) and 11 were perforated or missing in four fertile clutches (22% predated clutches). The proportion of predated clutches did not differ significantly between the mixed and the fertile clutches, either at the middle or the end of the experiment (Middle: $\chi^2=2.2$, $df=1$, $P=0.14$. End: $\chi^2=0.9$, $df=1$, $P=0.33$).

Fungi colonized dead eggs in the field experiment (seven of the 19 mixed clutches (37%) had mycelia in the two dead eggs at the middle of the experiment), but did not appear to spread to and kill fertile eggs. At the first inspection, among the 19 mixed clutches (none was predated), only one (5%) contained one (1%) fertile egg that died; among the 16 fertile clutches that were not predated, seven (44%) contained a total of 11 (11%) fertile eggs that died. At the second inspection, among the 17 non-predated mixed clutches, 11 (65%) contained 26 (38%) fertile eggs that died; among the 14 non-predated fertile clutches, all (100%) contained fertile eggs that died, totaling 35 (42%). The proportion of non-predated clutches containing fertile eggs that died was lower in the mixed than in the fertile clutches, both at the middle and the end of the experiment (Middle: $\chi^2=7.3$, $df=1$, $P=0.01$. End: $\chi^2=6.1$, $df=1$, $P=0.01$). The average proportion of fertile eggs that died per clutch did not differ significantly between non-predated mixed clutches and non-predated fertile clutches, both at the middle and the end of the experiment (Mann-Whitney *U*-test: Middle: $z=1.9$, $P=0.06$. End: $z=0.7$, $P=0.50$).

There was no negative overall effect of the presence of two dead eggs within a clutch on the incubation success of the remainder fertile eggs. The proportion of clutches containing fertile eggs that were predated or died from other (undetermined) causes was in fact lower among the mixed than among the fertile clutches (Middle: $\chi^2=9.4$, $df=1$, $P=0.002$. End: $\chi^2=6.8$, $df=1$, $P=0.009$). The average proportion of fertile eggs that were predated or died per clutch was lower in the mixed clutches at the first inspection, but did not differ significantly

between the two treatments at the end of the experiment (Mann-Whitney *U*-test: Middle: $z=2.3$, $P=0.02$. End: $z=1.0$, $P=0.29$).

EGG INFERTILITY, FUNGAL INFECTION, AND PREDATION IN NATURE

Field estimates of egg infertility and of eggs infected by fungi or predated by ants were all low. Only one of 36 (2.8%) females from Lagoa Comprida and two of 67 (3.0%) females from Torre laid clutches containing infertile eggs, corresponding to one infertile egg out of 248 (0.4%) eggs and two infertile eggs out of 380 (0.5%) eggs, respectively. From a total of 132 unhatched eggs that were dug out from natural nests, 110 (83%) were alive, 17 (13%) were dead, and 5 (4%) were destroyed by ants. Only one of the 17 (6%) dead eggs was surrounded by mycelia. Regarding the 286 eggshells collected from lizard nests, 222 (78%) were from eggs that had hatched, 46 (16%) from eggs that had not hatched and 18 (6%) from eggs that were predated by ants.

DISCUSSION

The present study reinforces previous evidence that fungi can kill reptilian eggs (Fitch & Fitch, 1968; Tracy, 1980) and that dead eggs in a clutch promote clutch colonization by pathogenic soil mycobiota (Phillott & Parmenter, 2001*b*). In the laboratory experiment, fungi (*Fusarium* sp. and *Gliocladium* sp.) colonized both infertile eggs and fertile eggs that died during incubation and thereafter spread to and killed adjacent eggs. Infertile eggs were more costly to the clutch than non-viable fertile ones, corroborating the idea that the timing at which dead eggs become available for fungal colonization of the clutch determines the proportion of eggs destroyed (Phillott & Parmenter, 2001*b*). These results also support the hypothesis that oophagy and removal of dead/infected eggs from the nest among lizards that provide care to the clutch (Mitchell & Groves, 1993; Somma, 2003) is an anti-pathogenic mechanism (Groves, 1982; Somma, 1989). Although fungi have been reported to grow under the conditions of lizard

nests (e.g. Hecnar, 1994), the spread of pathogenic fungi from dead to live eggs has not earlier been established in lizards.

Iberian rock lizards hatched earlier (before using the full egg yolk content) from fungal infected eggs than from non-infected ones. In fish and amphibians, early embryo hatching may be a behavioural strategy for escaping from eggs under the risk of pathogen infection (Warkentin *et al.*, 2001; Wedekind, 2002). This hypothesis, which remains to be tested in reptiles, implies that embryos respond to cues that stem from the pathogen or eggs, including embryos' alarm substances (Wedekind, 2002). Alternatively, fungal pathogens may induce early hatching by interfering with the natural mechanisms that trigger hatching (Warkentin *et al.*, 2001). As such, the present observations may be attributed to several causes, including fungal digestion of the eggshell (Ferguson, 1981), fungal exhaustion of the egg water content necessary for the incorporation of yolk into the embryo mass (Packard & Packard, 1988), and the arrest of yolk (precluding its use by the embryo) by hyphae that penetrate through the eggshell (Solomon & Baird, 1980). However, oxygen stress may provide the most parsimonious explanation for the observed results, as it triggers embryo hatching in several taxa (reviewed by Warkentin *et al.*, 2001) and possibly also in reptiles (Losos *et al.*, 2003). Moreover, fungi have been suggested to limit the oxygen available for sea turtle embryos by reducing the egg surface available for respiratory gas exchange (Phillott & Parmenter, 2001a). Iberian rock lizards that hatched earlier from infected eggs were possibly less fit, as amphibian embryos that hatched earlier (under predation or pathogen infection; Warkentin, 1995, 2000; Warkentin *et al.*, 2001) showed reduced survivorship (Warkentin, 1999). Side-blotched lizards (*Uta stansburiana*) that hatched from eggs that had some of the yolk removed were also smaller and suffered lower survivorship (Sinervo *et al.*, 1992).

The results from the field experiment did not corroborate the fungal pathogenic effects observed under laboratory conditions. The discrepancy between the two experiments may be attributed to two major causes: (1) different incubation conditions and (2) different quality of the eggs. The laboratory incubation conditions may have been optimal for fungal growth, allowing fungi to attain a degree of virulence not common in nature. The eggs used for the laboratory experiment were likely to be of lower genetic quality, as they were obtained from females that copulated fewer times and with fewer males than observed in nature and which were randomly allocated to males (preventing female mate choice). In contrast, eggs used for the field experiment were obtained from females that copulated in the field. Iberian rock lizard females are sexually promiscuous and possibly increase the genetic quality of their offspring through sperm competition, as documented for other reptiles (Olsson & Madsen, 2001). Moreover, female mate choice is described for this species and may result

in genetic benefits for the offspring (Martín & López, 2000; López *et al.*, 2002, 2003). Lower embryo genetic quality for the laboratory experiment might explain the large proportion of fertile clutches that contained non-viable fertile eggs and could have also led to high embryo susceptibility to fungal infections. In agreement with this, genetics (family identity) determined offspring resistance to viral pathogens in common lizards (*Lacerta vivipara*; Uller *et al.*, 2003). Prevention of female mate choice was directly implicated in reduced embryo resistance to bacterial pathogens in whitefish (*Coregonus* sp.; Wedekind *et al.*, 2001, 2004).

Taken together, the results from the present study do not conclusively demonstrate that dead eggs within Iberian rock lizard clutches compromise the incubation success of the remaining eggs. Even though clutches containing infertile or non-viable fertile eggs were colonized by fungi that killed eggs and reduced offspring quality in the laboratory experiment, such effects, or increased clutch predation, were not confirmed in the field experiment. Moreover, low field estimates of egg infertility, egg fungal infection and egg predation indicate that the scope for the above effects to operate in nature may be limited in the studied species. Nevertheless, the above hypothesis deserves further investigation in reptiles, as the laboratory results suggest a potential role of fungal pathogens in the selection of reproductive traits (Olsson & Madsen, 1998) that determine the probability of females laying infertile or non-viable fertile eggs and in the evolution of female egg-laying behaviour (e.g. choice of nesting sites that minimize fungal infection; physical separation of eggs within a nest or between nests). Good models for such studies could be provided by species, such as the sea turtles, that show a significant proportion of failed eggs in natural nests and that suffer from frequent nest contamination by fungi (Phillott & Parmenter, 2001b).

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THE REPRODUCTIVE BIOLOGY OF *SALAMANDRINA TERDIGITATA* (CAUDATA, SALAMANDRIDAE)*

FRANCESCA DELLA ROCCA, LEONARDO VIGNOLI AND MARCO A. BOLOGNA

Dipartimento di Biologia, Università "Roma Tre", Roma, Italy

We studied the reproductive biology of the spectacled salamander, *Salamandrina terdigitata*, in Central Italy by daily sampling over three breeding seasons. Reproduction takes place annually, between February and May. Clutch size varies (1-65), and are usually placed by females on the underside of stones. The total number of eggs deposited varies yearly and is positively related to the period of preceding rainfall. Large females start breeding earlier and show stronger oviposition site fidelity than small ones. For oviposition females choose the portions of the stream that have the highest density of stones. The speed at which embryos develop increased over time, presumably under the influence of rising water temperature. Hatching success is negatively affected by aquatic drift, desiccation, predation by Trichoptera larvae and the absence of hiding sites.

Key words: egg production, Italy, microhabitat selection, salamander

INTRODUCTION

The main limitation of studies on the reproductive biology of species characterised by short individual reproductive periods, is the lack of data due to low sampling frequency. The spectacled salamander, *Salamandrina terdigitata* (Lacépède, 1788), is an elusive species, in which the reproductive biology is rarely studied. Each female has a brief aquatic phase, limited to oviposition activity.

Salamandrina terdigitata belongs to a monotypic genus that is endemic to the Italian peninsula (Lanza, 1988), representing one of the oldest branches within the Salamandridae, with no close Palaearctic relatives (Titus & Larson, 1995). The range of the species extends from central Liguria (Genoa Province) to southern Calabria (Reggio Calabria Province), primarily along the Tyrrhenian slope of the Italian peninsula, with most occurrences at 200-700 m a.s.l. (Societas Herpetologica Italica, 1996; Vanni & Nistri, 1997; Zuffi, 1999; Corsetti & Angelini, 2000). It is considered threatened by the EU Habitats Directive and the Berne Convention. *Salamandrina terdigitata* is a largely terrestrial species adapted to submesic or mesic forests. It can also be found in mixed landscapes composed of pastures and residual woodlands or other derived habitats. For oviposition *S. terdigitata* uses well oxygenated streams with low or laminar flow, stony bottoms and dense riparian vegetation, even if small artificial water basins are utilised (Vanni, 1980; Lanza, 1983; Corsetti, 1999a; Barbieri, 2001; pers. obs.).

The aim of the present research is to study several aspects of the reproductive biology of this species in depth, such as microhabitat selection and oviposition phenology in relation to habitat parameters, the number of eggs deposited, the reproductive success and development rate.

To date only scarce information on this topic, based on occasional records is available and there have been few attempts to investigate the reproductive behaviour and phenology of this species (Ramorino, 1863; Strötgen, 1927; Naviglio, 1971; Barbieri & Tiso, 1993; Corsetti, 1999a,b; Angelini *et al.*, 2001; Vignoli *et al.*, 2001a).

MATERIAL AND METHODS

STUDY AREA

The population of this study is situated in a natural area inside Rome, in the Insugherata Natural Reserve, at 80 m a.s.l. It is restricted to a very small valley of about three hectares in surface area, with mesic woodland dominated by *Castanea sativa* and *Ostrya carpinifolia*, along a short perennial and sub-rectilinear stream, tributary of the Fosso dell'Acqua Traversa, which flows into the Tiber River. The Reserve, which covers an area of about 700 hectares, is a mosaic of weed cultivations, pastures, thermophilic woodlands, and small marshes with riparian hygrophilic woodlands.

SAMPLING METHODS AND STATISTICAL ANALYSIS

The study period extended from February 1999 and April 2002. Surveys were carried out exclusively within the oviposition site, which is represented by a portion of a stream, about 110 m in length, from the source to a small waterfall (3 m in height). Surveys were extended to the entire stream (about 400 m in length) and to other streams of the Reserve, but no salamanders were found. Although the sexes are indistinguishable morphologically (Brizzi *et al.*, 1989), only females are aquatic during the short oviposition phase (Lanza, 1983), and consequently, all specimens found in the water were considered to be females.

Sampling was carried out every day in spring, less frequently in autumn and winter, as well as in June.

Correspondence: M. A. Bologna, Dipartimento di Biologia, Università Roma Tre, Viale Marconi, 446, I-00146 Roma, Italy. *E-mail* : bologna@bio.uniroma3.it

The sampling period was considered to range from the first to the last oviposition found. To define the oviposition period, the study area was checked every year from February to mid-May. Sampling carried out in 1999 was restricted to the identification of each specimen using ventral photography of egg-laying salamanders. The research in 2002 stopped before the end of the oviposition activity, hence we used this data set only for some aims (microhabitat selection, distribution along the stream, correlations between biometry and reproductive biology).

Each substrate available for oviposition was numbered and its position was recorded at every sampling. The oviposition site was divided into 11 sections (A-M), each 10 m in length, starting from the source. Every season, the map was modified according to any morphological variations in the stream, due to rain, flow and erosion. Sections of the stream were divided into two main groups based on topography. The first area, including sections A and B, is characterised by high, sloping (about 70°-90°) banks, mostly shady because of the overhanging vegetation. The second area (sections C-M) has slightly sloping banks, with scattered overhanging vegetation, and it is more exposed to the sun. Multi Response Permutation Procedure test (MRPP), applied to the female size (mouth-cloaca length: MCL), showed a lower intra-group variance (A-B and C-M) than among sections ($P < 0.05$), supporting the division of the stream in two homogeneous areas.

Each sampling in the 2000-2002 period included both physical and biological surveys. We recorded the water temperature using a multiparametric probe. We tested the relationship between the number of available substrates for oviposition and the presence of salamanders among the stream sections in which we found specimens. Each captured specimen was recorded by a photograph of the ventral pattern and a photographic database was prepared. Using an electronic calliper (resolution 0.01 mm) we measured the body length, from apex of head to middle of cloaca (MCL) of each specimen. We recorded body measurements when spectacled salamanders were first captured, and repeated the measurements every year, at each first recapture. An analysis of female fidelity to the oviposition site was carried out on individuals recaptured at least once during the study period: consequently we considered only females captured in 2000 and then recaptured in 2001. The daily sampling allowed us to assess in several cases the egg number in a single egg mass laid by a female. The daily variation in the number of eggs and masses in the oviposition area depended on three main factors: egg deposition rate, embryonic mortality and hatching. We also evaluated the embryonic survival rate and number of eggs hatched. Lastly, we recorded the number and phenology of larvae during the sampling period.

Statistical analysis was performed using the package STATISTICA 5.1 G (1997, Statsoft) and the Microsoft Excel 2000 programme. Descriptive statistics, both parametric (t -test to compare the size of females) and

non-parametric (Mann-Whitney U -test, to compare the presence of females and stones in the stream; Chi-square test to examine the female fidelity to the stream sections; Spearman correlation to test if females enter the water in the same sequence each year), and linear regression (to relate the development rate to water temperature), were utilised to evaluate results.

RESULTS

REPRODUCTIVE PHENOLOGY

During the sampling period we recorded 165 female spectacled salamanders: 36 in 1999, 60 in 2000, 57 in 2001, 12 in 2002. The population had a single spring reproductive period, extending from February to mid May, with great variation between years. The oviposition period extended from 20 March to 26 April in 1999; from 1 March to 6 May in 2000; from 13 February to 13 May in 2001; and from 9 March to 18 April in 2002.

Females usually enter the water only once to lay eggs during one reproductive season. Only 13 specimens came back to the stream after their first visit within the same year: 6 out of 74 (8.1%) in 2000; 6 out of 99 (6.1%) in 2001; 1 on 31 (3.2%) in 2002. Females remain in the water for between one and eight days (2000: mean = 2.85; $n=74$; $SD=1.91$; 2001: mean = 2.47; $n=98$; $SD=1.73$).

An analysis based on 32 individuals captured in 2000 and recaptured in 2001 highlighted a significant correlation between years for individual time of entrance at the oviposition site ($n=32$; $r=0.66$; $P < 0.001$; Spearman correlation; Fig. 1).

MICROHABITAT SELECTION

The number of specimens recorded from 2000 to 2002 in each section (A-M) of the stream is reported in Table 1. The 2000 oviposition area was restricted to the first two sections, A-B, particularly in B. In 2001 the oviposition area was greatly expanded and sections A-C, E-H and M were colonised; sections A and B (particularly A) were utilised more than others. In 2002 the number of ovipositions greatly decreased because of

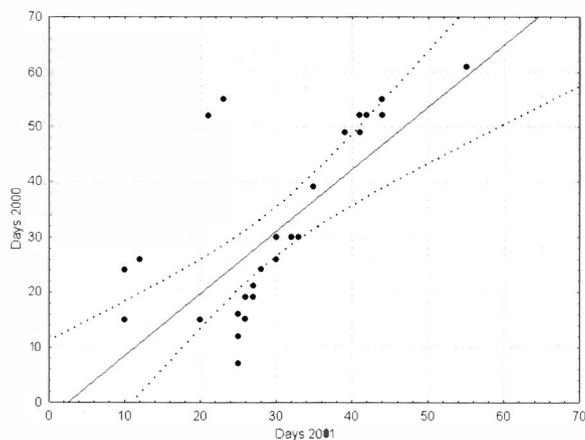


FIG. 1. Spearman correlation of the order of entrance in water of 32 specimens captured in 2000 and recaptured in 2001. Circles represent individuals. For both years the first day of oviposition was indicated as day 1.

TABLE 1. Distribution of specimens and available stones for oviposition in each stream section (2000-2002). Some specimens (8 in 2000 and 11 in 2001) visited both sections A and B during the same reproductive period; hence the sum of the respective columns exceeds the real number of the observed specimens.

Stream sections	2000		2001		2002	
	Specimens	Stones	Specimens	Stones	Specimens	Stones
A	32	24	54	20	16	16
B	50	34	45	21	6	9
C		5	1	1		-
D		-		-		2
E		10	2	14		10
F		7	4	5		3
G		4	1	4	9	3
H		6	2	5		4
I		-		-		-
L		-		-		-
M		2	1	1		-

a reduced water flow, which uniformly affected the entire stream; in this year only sections A-B and G, where submerged objects for oviposition remained available, were utilised.

The 29 females captured in 2000 and recaptured in 2001 tended to lay eggs in the same section (A and B) of the stream as in the previous year ($n=29$; $df=1$; $\chi^2=7.60$; $P<0.01$). The females ovipositing in the two areas differed in size (Table 2): females that oviposited in sections C-M were smaller than those using sections A-B, both in 2001.

Three different substrates were available to oviposit in the stream: stones, leaves or other plant parts, and artificial (plastic or metallic) supports. Water depth was not a significant factor for oviposition: females laid eggs from the water surface to the maximum depth observed in the stream. The salamanders more frequently used stones than other objects for oviposition (Table 3). In 2002 a greater variability in substrate selection was observed, probably due to the reduction of available stones, because of the winter drift. In that year, the salamanders also used some tracts of the stream without stones for oviposition. These contained numerous leaves or tree branches, amassed in small bends, at least 10 cm in depth.

TABLE 2. Difference in size (snout-vent length, SVL) between specimens ovipositing within tract A-B (78 in 2001, 21 in 2002) and those ovipositing within tract C-M (13 in 2001, 7 in 2002) (2001-2002).

	Mean _{A-B}	Mean _{C-M}	<i>t</i>	df	<i>P</i>	SD _{A-B}	SD _{C-M}
SVL 2001	3.77	3.44	3.52	89	0.00069	0.32	0.29
SVL 2002	3.76	3.47	2.97	26	0.0063	0.21	0.25

TABLE 3. Number of specimens found under each substrate (2000-2002).

Substrate	No. of specimens (2000)	No. of specimens (2001)	No. of specimens (2002)
Stones	66 (89.19%)	93 (93.94%)	22 (70.97%)
Leaves	8 (10.81%)	5 (5.05%)	7 (22.58%)
Others	-	1 (1.01%)	2 (6.45%)

Females oviposited on the underside of stones of variable size, but with a lower surface area of 90-540 cm². The number and distribution of stones varied both during the study period and within the same season, because of stream drift due to the intensity of rainfall. There is a significant relationship between the presence of salamanders and stone availability (Table 1): based on data of 2000 and 2001, the ratio between the number of specimens and available stones in the first (A-B sections) and second (C-M sections) areas of the stream 1.91 and 0.6m respectively, differ significantly (Mann-Whitney *U*-test: $n_{A-B}=4$, $n_{C-M}=6$; $U=0$; $P<0.05$).

The females laid eggs by sticking them to the underside of the stones or, rarely, on other objects (leaves, tree branches, artificial supports). The percentage of available stones utilised varied between years, from 16 out of 47 (34%) in 2002 to 47 out of 71 (66%) in 2001.

NUMBER OF EGGS

Eggs were usually deposited very close to each other, forming an egg mass of variable size (Fig. 2). Each female laid eggs in separate masses under a single or several substrates, as noted by direct observation. The number of eggs oviposited over a single reproductive season varied each year and within the two areas (A-B

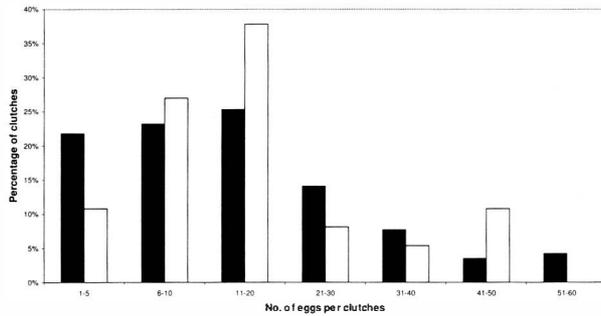


FIG. 2. Percentage distribution of clutches depending on number of eggs per clutch (black, 2000; white, 2001).

and C-M): Fig. 3 indicates the number of eggs per day, year and section. In the first area (sections A-B), this number is comparable both in 2000 and 2001 (respectively 2462 and 2495), while the second area (sections C-M) has been utilised for oviposition activity only since 2001 (850).

The peaks of egg-laying activity varied in time and quantitatively each year: e.g., 1 May 2000, 1201 eggs, at the end of the reproductive season; 20 March 2001, 1428 eggs, in the first area, and 10 April 2001, 604 eggs, in the second area; in 2002, the maximum number of eggs observed before the samplings stopped was 320 (17 April) in the first area, and 89 (20 April) in the second area. In 2001 the oviposition activity started in the second area on 2 April, when 1072 eggs were already present in the first area. The decrease in the rate of oviposition of new eggs in the first area almost coincides with the increased oviposition activity in the second area. In 2002, oviposition again started in the first area (9 March) before the second (15 April). The average egg number per egg mass in 2000 was 16.86 ($n=140$; $SD=14.48$; range 1-65), and in 2001 was 16.41 ($n=36$; $SD=11.64$; range 1-44).

EGG DEVELOPMENT AND SURVIVAL

The embryo development period varies from a maximum of 37 days (eggs oviposited at the beginning of the

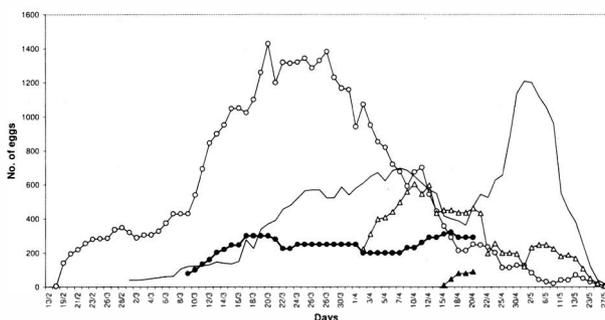


FIG. 3. Egg numbers during three reproductive seasons (2000-2002). Time-course of eggs is determined by the computation of egg deposition rate, embryo mortality and hatching. The A-B area is represented in 2000 by smooth line, in 2001 by empty circles, in 2002 by solid circles. The C-M area is represented in 2001 by empty triangles and in 2002 by solid triangles.

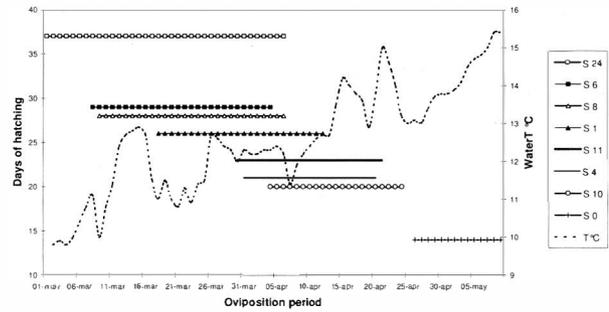


FIG. 4. Relation between hatching period of sampling egg masses (S24-S6-S8-S1-S11-S4-S10-S0) and water temperature.

season) to a minimum of 12 days for the latest oviposition. The duration seems to be greatly influenced by water temperature, as higher water temperature reduces the rate of embryonic development. Eight ovipositions from different sites are compared in Fig. 4: S24 hatched after 37 days at a mean temperature of 11.65°C ($SD=0.86$); S6 and S8 respectively after 29 and 28 days, at a mean temperature of 11.68°C ($SD=0.78$) and 11.76°C ($SD=0.77$); S0 after 14 days at an average temperature of 14.17°C ($SD=0.79$). A linear relation between water temperature and time of hatching emerged from an analysis based on the data set of eight egg masses (linear regression: $y=-6.96x+111.33$; $r^2=0.81$; $P<0.005$).

The average percentage of hatched eggs in two sampling years (2000 and 2001) was 55.61% (2000: 1286 out of 2462 = 52.23%; 2001: 1973 out of 3345 = 58.98%).

Larvae were only observed in the first days after hatching. They usually drifted to different parts of the stream, or hid under leaves or rubble.

DISCUSSION

The single spring oviposition period characterising this and other studied populations of spectacled salamander (Zuffi, 1999 for a review) is probably due to the perennial condition of the stream. Some Central Italy populations occurring in temporary waters have a second autumnal reproductive season, as noted by Corsetti (1999a,b) and Angelini *et al.* (2001).

The salamanders used mainly one area of the stream (sections A-B) for oviposition, probably because of the larger number of available substrates and the greater general protection (vegetation, banks structure, etc.). Three main factors concurred to extend the oviposition area from the first area (1999-2000) to the second one (2001-2002): (1) hydromorphological modifications within sections C-M which made this area suitable for oviposition from 2001; (2) winter drift phenomena, which decreased the number of potential substrates for laying eggs in the first area, and increased these substrates in the second area; (3) increased population size, observed from 1999 to 2001 (about 100% each year; triple catch index) (Della Rocca *et al.*, unpublished).

Larger females showed a clear fidelity towards the oviposition site (A-B). Moreover, the tendency of the significantly shorter specimens, presumably younger animals, to reach the stream after the older ones (observed in other populations by Angelini *et al.*, 2001), could force the former individuals to look for available substrates for oviposition out of sections A-B. Our results suggest that the most used substrate for egg laying is the underside of stones, even though not all available stones were utilised. These results differ from those obtained by other authors (e.g. Lanza, 1983; Zuffi, 1999; Barbieri, 2001), who considered other objects as the most used substrate. Personal observations on other populations in Central Italy (e.g. Tolfa Mts., Sabini Mts., Reatini Mts.) indicate that oviposition substrates vary according to local availability. Even though stones are the most suitable substrates for oviposition, females of this population showed great plasticity when the availability of the preferred substrate decreased (e.g. in 2002), using a variety of alternative substrates such as leaves or submerged branches.

Each female oviposited one or more egg masses, consisting of a variable number of eggs, or less frequently a single egg, each glued to the substrate by a peduncle. This observation agrees with much of the literature (e.g. Ramorino, 1863; Thorn, 1968; Angelini *et al.*, 2001), but diverges from Lanza (1983), who considered egg groups to be the result of adhesion of eggs deposited by several females. The number of eggs per mass is comparable with literature records (e.g. Bruno, 1973; Zuffi, 1999).

The number of oviposited eggs is comparable in the first area (A-B) in both 2000 and 2001, while in the second one (C-M, used since 2001) the number varied. The first area seems to reach regularly its carrying capacity for the oviposition substrates, but the second one seems to be utilised only when habitat conditions are suitable.

Embryo development varied between 12 and 37 days, and is positively related to water temperature, as reported in literature (Corsetti, 1999a,b). Both biotic and abiotic causes influenced the embryo mortality. Abundant rain caused stones and other substrates to drift, either crushing the eggs or exposing them to air. On the other hand, scarcity of rain decreased water levels, producing desiccation or anoxic conditions. Moreover, banks that collapsed caused changes to the stream structure with analogous effects. The main biotic cause of mortality is represented by predation on the eggs by the larva of the trichopteran *Plectrocnemia conspersa* Curtis, 1834 (see Vignoli *et al.*, 2001b). The predator crab *Potamon fluviatile* Herbst, 1785, observed feeding on a dead adult specimen (see also Lanza, 1983), may represent a further threat.

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After the acceptance of this manuscript, using mtDNA analysis Mattocchia *et al.* (*Zootaxa* **995**, 1-19, 2005) demonstrated the existence of two species of spectacled salamander. The population studied in this paper belongs to *S. perspicillata* (Savi, 1821).

TROPHIC EGGS IN THE FOAM NESTS OF *LEPTODACTYLUS LABYRINTHICUS* (ANURA, LEPTODACTYLIDAE): AN EXPERIMENTAL APPROACH

CYNTHIA P. A. PRADO, LUÍS FELIPE TOLEDO, JULIANA ZINA AND CÉLIO F. B. HADDAD

Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil

The South American pepper frog, *Leptodactylus labyrinthicus*, is a large species that lays eggs in foam nests in holes dug out of the banks of different bodies of water. Recently, it was reported that only 6-10% of eggs are fertilized in foam nests of *L. labyrinthicus* and the remaining unfertilized eggs are consumed by the tadpoles inside the nest. Here we tested experimentally the influence of the ingestion of trophic eggs on the survivorship and growth of *L. labyrinthicus* tadpoles. Tadpoles fed on trophic eggs and subsequently fed on dry fish food grew larger than those fed only on dry fish food, and this suggests that the ingestion of trophic eggs is an adaptation to improve tadpole growth. The ingestion of trophic eggs also seems to be important for the maintenance of tadpoles in environments with unpredictable rainfall, as they were able to survive for about 70 days feeding only on these trophic eggs and one tadpole managed to complete metamorphosis feeding on trophic eggs only. Details of the spawning behaviour observed in the field, occurrence of multiple mating, and predation on eggs by terrestrial invertebrates and vertebrates are also reported.

Key words: egg predation, multiple mating, frog, reproduction, spawning behaviour

INTRODUCTION

The genus *Leptodactylus* is characterized by the deposition of eggs embedded in foam nests (Heyer, 1969), showing an evident tendency toward a terrestrial existence, i.e. reproduction becoming gradually independent of water (Heyer, 1969). Studies on *Leptodactylus* published since the revision made by Heyer (1969), report several novelties in the reproductive modes of the frogs in this genus (e.g. Hero & Galatti, 1990; Davis *et al.*, 2000; Prado *et al.*, 2002). For the *L. pentadactylus* group, with approximately 13 species (Frost, 2004), two different reproductive modes have been described. One of them is the mode described by Heyer (1969), with foam nests deposited in depressions or holes close to water and exotrophic tadpoles that develop in water (e.g. *L. knudseni*: Hero & Galatti, 1990; Rodríguez & Duellman, 1994; *L. labyrinthicus*: Agostinho, 1994; Rodrigues Silva *et al.*, 2005). A totally terrestrial mode in the group was reported for *L. fallax* (Davis *et al.*, 2000) and *L. pentadactylus* (Hero & Galatti, 1990; Rodríguez & Duellman, 1994), with foam nests inside burrows in the ground and development of larvae inside the nest. At least for *L. fallax*, it was recently reported that tadpoles display obligatory oophagy, feeding only on trophic eggs deposited by the females (Gibson & Buley, 2004), as previously suggested by Prado *et al.* (2002).

Although the presence of a foam nest has been widely reported for leptodactylines, foam functions are still scarcely understood (Downie, 1993). Many functions are attributed to foam nests made by several anuran species, namely, defence against predators (Kluge, 1981; Ryan,

1985; Downie, 1988; 1990; 1993), temperature control (Downie, 1988), protection against desiccation (Ryan, 1985; Downie, 1988), inhibition of tadpole growth (Pisano & Del Rio, 1968), improvement of oxygen supply (Seymour & Loveridge, 1994), and food source (Vinton, 1951; Tanaka & Nishihira, 1987). Tadpoles of different *Leptodactylus* species in the *Pentadactylus* group have been recorded feeding on foam in the nest (*L. pentadactylus*: Vinton, 1951), as well as on conspecific eggs inside the same nest (*L. pentadactylus*: Muedeking & Heyer, 1976; *L. labyrinthicus*: Agostinho, 1994), allowing tadpoles to survive for long periods before being washed to the water by rain.

The South American pepper frog, *Leptodactylus labyrinthicus*, is a large species that lays eggs in foam nests in holes dug out on the banks of different bodies of water (Rodrigues Silva *et al.*, 2005), occurring throughout South America (Heyer, 1979). However, its reproductive biology is still poorly known (e.g. Cardoso & Sazima, 1977; Agostinho, 1994; Rodrigues Silva *et al.*, 2005). Recently, it was reported that only 6-10% of eggs are fertilized in the foam nests of *L. labyrinthicus* (Agostinho, 1994; Rodrigues Silva *et al.*, 2005), and the remaining unfertilized eggs are consumed by the tadpoles inside the nest. Based on this information, we tested in the laboratory the influence of the ingestion of trophic eggs on the survivorship and growth of *L. labyrinthicus* tadpoles. As the species lives in environments with unpredictable rainfall and may remain in the nest for more than 30 days (Agostinho, 1994; Eterovick & Sazima, 2000b), we tried to determine the importance of the trophic eggs in the diet of *L. labyrinthicus* tadpoles by simulating field conditions to answer the following questions: (1) do tadpoles remaining in the foam nest and feeding on trophic eggs grow larger than those that leave the foam

Correspondence: C. P. A. Prado, Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Caixa Postal 199, 13506-970, Rio Claro, São Paulo, Brazil.
E-mail: cpap@rc.unesp.br

prematurely? And (2) how long can the tadpoles survive by remaining in the foam nest and feeding only on trophic eggs? Furthermore, details of the spawning behaviour observed in the field, multiple mating, and predation on eggs by terrestrial invertebrates and vertebrates are reported.

MATERIALS AND METHODS

SPAWNING BEHAVIOUR AND PREDATION EVENTS

Field observations were made at the Estação Ecológica de Itirapina (EEI), between the municipalities of Brotas and Itirapina (22°13'S; 47°54'W), State of São Paulo, south-eastern Brazil. This area represents one of the last remnants of Cerrado in the State of São Paulo, which is an open formation covered by savannah-like vegetation. Monthly field observations were made during the day and at night in two distinct periods: October 2002 through February 2003 and October 2003 through January 2004, which correspond to the breeding period of this species at the site. Individuals of *L. labyrinthicus* were observed breeding at the edge of temporary ponds and small streams. We used all occurrences sampling (Altmann, 1974) to describe spawning and nest excavation behaviours. All foam nests observed were checked for predators.

TADPOLE GROWTH AND SURVIVORSHIP

Foam nests of *L. labyrinthicus* were collected at the Estação Ecológica de Itirapina (EEI: 22°13'S; 47°54'W) and in the municipality of Rio Claro (22°24' S; 47°33' W), State of São Paulo, south-eastern Brazil, between October - December 2001 and October-December 2002. When collected, clutches contained tadpoles at stages 18 and 19 (Gosner, 1960); total number of eggs and percentage of fertilized eggs were recorded. For the experiments, tadpoles were kept in the foam nests until they reached 6.0 mm of body length (stage 25; Gosner, 1960). All foam nests and tadpoles were maintained in the laboratory in plastic boxes (30 x 40 cm) with 3.0 cm of well water, with water temperature between 25 and 27°C (mean temperatures registered in the field), and natural photoperiod (sunlight coming through the windows). As tadpoles of *L. labyrinthicus* are carnivorous (Eterovick & Sazima, 2000a), pellets of dry fish food (Alcon®) were used in the experiment as the alternative food source.

To test for differences in growth rate and survivorship, tadpoles were divided into three groups: Treatment I, 25 individuals ($N=3$ clutches) reared in foam nests with trophic eggs, and subsequently fed dry fish food pellets *ad libitum* once a day; Treatment II, 33 individuals ($N=3$ clutches) reared in water and fed only dry fish food *ad libitum* once a day; Treatment III, groups of 14 and 20 tadpoles ($N=2$ clutches) were reared in foam nests with trophic eggs, without additional food. The body length of 10-20 larvae was measured for each treatment at intervals of about five days with a calliper ruler to the nearest 0.1 mm. Snout-vent length (SVL) of metamorphosed specimens was recorded with a calliper

ruler to the nearest 0.1 mm and body mass was measured on an electronic balance to the nearest 0.01 g. Some tadpoles and metamorphs were deposited at the Célio F. B. Haddad collection (CFBH 5879-5902), in the Departamento de Zoologia, Universidade Estadual Paulista, Rio Claro, São Paulo, Brazil, but most metamorphs were released at the site where they had been collected after being health-screened.

RESULTS

SPAWNING BEHAVIOUR

On 9 January 2004 at the Estação Ecológica de Itirapina, we recorded three amplexant pairs building foam nests. Spawning behaviour was similar in the three nests, the main steps being summarized as follows. Males called from shallow natural cavities next to lentic water bodies, or from old hollows that had already been used for foam nest deposition. The female approached a calling male, which began to emit courtship call. The male clasped the female's flanks; the female arched its body down and discharged a jelly-like secretion throughout its vent. At this moment, the male started the foam production by stirring the secretion with its hind limbs. Next, the female pushed the mud with its arms from the centre to the edges of the nest, digging it deeper. When the female's movements ended, the couple rotated about 20° to their right. It took approximately 19 short turns to complete 360° in the nest, which lasted about 14 min. This behaviour was repeated until the end of the foam nest construction. Nest excavation by the female and spawning occurred simultaneously and lasted about three hours, starting between three and four hours after sunset. We observed that the eggs were not laid at the beginning of the foam construction, but only when the foam reached a larger size. In all three cases, males left the nest before the females, which stayed inside the nest for up to another 10 min.

On the same night we observed two nests in which three individuals tried to build the foam nest in a hollow, all at the same time. In one case it was a multimale spawning (two males and one female) and in the other, a multifemale spawning (two females and one male). We do not know the moment when the multimale group formed. However, we observed that the multifemale group formed when a second female entered a nest between the amplexant pair. The presence of a third individual in both nests made the foam production movements awkward. Males could not move their legs properly and females could hardly move in the nest or even push the mud forward. The multiple mating groups remained together for up to three hours, trying to raise a foam nest; suddenly they gave up and left the nests without spawning.

PREDATION

Predation within the nests was recorded several times at the Estação Ecológica de Itirapina. On 2 December 2003 at one temporary pond edge, ants (*Camponotus rufipes*, Formicinae) were observed preying upon *L.*

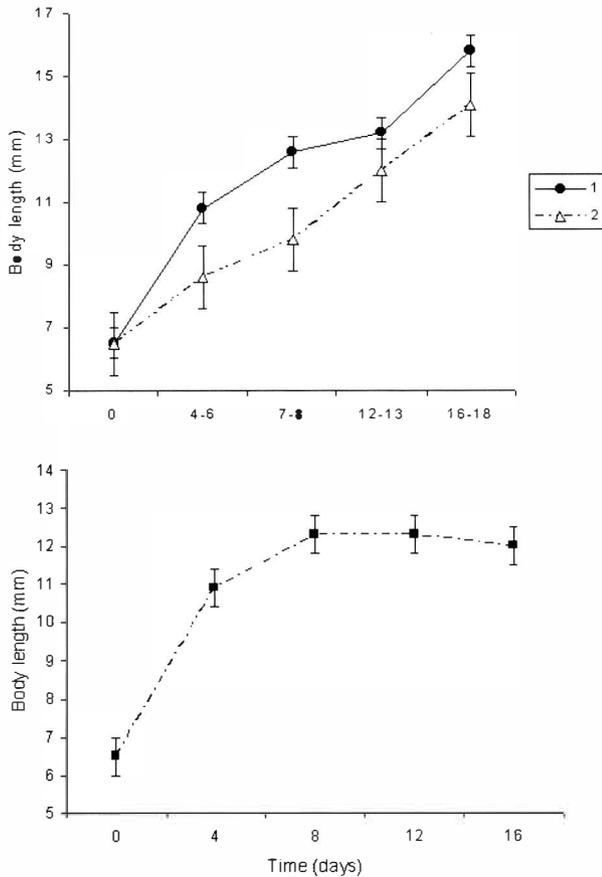


FIG. 1. (A) Mean body length of *Leptodactylus labyrinthicus* tadpoles reared (1) in foam nest with trophic eggs, and subsequently fed on dry fish food (solid circles; $N=15$); and (2) in water fed only on dry fish food (open triangles; $N=20$). The arrow indicates the time when tadpoles changed their diet to dry fish food. (B) Mean body length of tadpoles ($N=15$) reared in foam nest with trophic eggs but no additional food. The arrow indicates the time when the trophic eggs finished; Bars = 1 SD.

labyrinthicus eggs that were on the outermost layer of the foam nest. The ants pulled the eggs from the foam with the mandibles and carried them away. On 9 January 2004 at another pond, two other nests were also being preyed upon by the same ant species. On 2 December 2003, a colubrid snake was observed in a *L. labyrinthicus* foam nest that contained tadpoles. The snake fled before we could catch it and thus its identity remains unknown.

On several occasions throughout the study period, some foam nests of *L. labyrinthicus* were destroyed during the night, but the predator was never seen. On 9 January 2004, one foam nest was found at 19.20 hr. At 20.00 hr, an adult maned wolf (*Chrysocyon brachyurus*) was seen at a temporary pond next to the foam nest (N.L. Hulle, pers. comm.). At 21.30 hr, we returned to the pond and found only 10% of the foam nest in a ruined cavity where footprints of *C. brachyurus* were found. At the same pond, 2 m away from recent wolf faeces we found a forelimb and parts of the abdomen (with ovaries and mature oocytes) of an adult female *L. labyrinthicus*. We presume the wolf ate the frog since these body parts had not been seen in the pond beforehand.

TADPOLE GROWTH AND SURVIVORSHIP

Foam nests contained an average of 2756 ± 821 eggs ($N=4$; range=1817 - 3786), and the mean (\pm SD) percentage of fertilized eggs was $6.5 \pm 3.0\%$ eggs ($N=5$; range=3.4-11.5%). Individuals reared in foam nests with trophic eggs and subsequently fed on dry fish food (Treatment I) also consumed the foam and grew larger than those reared in water and fed only on dry fish food (Treatment II; Fig. 1A). All larvae from Treatments I and II reached metamorphosis within about 40 days from the date they were collected (clutch about 3-5 days old). Mean (\pm SD) SVL of metamorphs from Treatment I was 23.0 ± 1.4 mm ($N=25$; range=20.1-25.8 mm) and mean (\pm SD) mass was 1.38 ± 0.2 g ($N=23$; range=0.83-1.77 g). From Treatment II, metamorphs reached a mean (\pm SD) SVL of 21.5 ± 1.5 mm ($N=33$; range=18.4-24.8 mm) and a mean (\pm SD) mass of 1.17 ± 0.2 g ($N=31$; range=0.78-1.5 g). Metamorphs from Treatment I were significantly larger ($t=3.6$, $df=56$, $P=0.0006$) and heavier ($t=3.5$, $df=52$, $P=0.001$) than those from Treatment II. Tadpoles reared in foam nests with trophic eggs but no additional food also consumed foam (Treatment III), but these shrank and their development slowed down after 12-13 days (Fig. 1B), when the trophic eggs and foam finished. However, some of them survived for about 40 days ($N=6$ from 14 tadpoles) in the first clutch tested, and even for about 70 days ($N=18$ from 20) in the second. Among these tadpoles, only one reached metamorphosis after 34 days, but with a small size (SVL=15.8 mm; mass=0.4 g).

DISCUSSION

Despite its wide geographical distribution, the spawning behaviour of *L. labyrinthicus* and the gender responsible for nest excavation remained unknown until the present study and the study carried out by Rodrigues Silva *et al.* (2005). Our observations on nest excavation by the amplexed female and foam construction are consistent with those described by Rodrigues Silva *et al.* (2005) for another *L. labyrinthicus* population. Furthermore, this is the first report of multiple mating for the species. Multimale spawning was reported for *Leptodactylus chaquensis* and *L. podicipinus* (Prado & Haddad, 2003), as well as for other species in some other families (e.g. Kusano *et al.*, 1991; Roberts *et al.*, 1999). In multimale breeder species, males have much larger relative testes size compared with other species, probably a trait related to sperm competition (e.g. Kusano *et al.*, 1991; Jennions & Passmore, 1993; Prado & Haddad, 2003). However, males of *L. labyrinthicus* have much smaller testes mass relative to body mass compared with other leptodactylids (Prado & Haddad, 2003) and we suggest that this could be related to the fact that males do not need to produce a great amount of sperm, since only about 10% of eggs are fertilized. Furthermore, polyandry seems to be accidental in this species since it prevents egg laying. Occurrence of simultaneous polygyny, as observed for *L. labyrinthicus*

in the present study, is unrecorded for frogs as far as we know. The trios observed in the hollows (multifemale and multimale) abandoned the foam construction before spawning, probably due to spatial limitation. We suggest that the explosive reproductive pattern allied to an almost 1:1 sex ratio (L.F. Toledo, unpublished data) of *L. labyrinthicus* could be related to the multiple mating behaviour observed at the study site.

The deposition of eggs outside the water is suggested to be an adaptation against aquatic predators (e.g., Magnusson & Hero, 1991; Haddad & Sawaya, 2000). For *L. labyrinthicus*, whose tadpoles are known to be carnivorous (Cardoso & Sazima, 1977; Eterovick & Sazima, 2000a), it has been suggested that the deposition of foam nests in depressions isolated from the water body might protect the tadpoles from predation by conspecific tadpoles (Rodrigues Silva *et al.*, 2005). Although nests of *L. labyrinthicus* may protect eggs, embryos, and tadpoles from aquatic predators, it seems to imply in an additional cost, as they are vulnerable to some terrestrial predators, both invertebrates and vertebrates (Menin & Giaretta, 2003; present study). Terrestrial invertebrates preying on eggs inside foam nests had already been reported for other *Leptodactylus* species, including those that place eggs inside subterranean chambers outside the water, such as *L. fuscus* and *L. latinasus* (e.g. Villa *et al.*, 1982; Downie *et al.*, 1995).

Our results confirm the occurrence of trophic eggs in the foam nests of *L. labyrinthicus*, as previously reported (Agostinho, 1994; Rodrigues Silva *et al.*, 2005). Histological study of the ovary, unfertilized eggs, and testis of *L. labyrinthicus* (Prado *et al.*, 2004) did not reveal any abnormality that could explain the low fertilization rate. However, it was observed that females remain in the nest after spawning (Rodrigues Silva *et al.*, 2005; present study), and thus the most plausible explanation for the low fertilization rate would be that females place additional eggs following spawning, as previously suggested by Rodrigues Silva *et al.* (2005). The observation that *L. fallax* females return to the nest in several occasions to place trophic eggs (Gibson & Buley, 2004) seems to support the hypothesis that females of *L. labyrinthicus* also deposit trophic eggs, since both species are phylogenetically related and are usually placed within the same species group (Heyer, 1979; Eterovick & Sazima, 2000a). The origin of carnivory in *L. labyrinthicus* tadpoles may be explained by a few simple behavioural steps, from the ingestion of trophic eggs in the nest (e.g. Agostinho, 1994; present study) to the predation on eggs and larvae of other frog species (e.g., Cardoso & Sazima, 1977; Rodrigues Silva *et al.*, 2005) going through cannibalistic behaviour by preying upon conspecific tadpoles and eggs (e.g. Rodrigues Silva *et al.*, 2005).

Among leptodactylids that build foam nests outside the water, several adaptations have evolved in order to maintain tadpole development, from production of additional foam by the tadpoles to remain in the nest waiting for rain (e.g. *Leptodactylus fuscus*: Downie, 1989) to the

deposition of eggs rich in yolk to nourish tadpoles that do not leave the nest (e.g. some *Adenomera* species: De la Riva, 1995). The deposition of a large amount of eggs that will not be fertilized, but instead will be consumed by the tadpoles, as recorded for *L. labyrinthicus* (Rodrigues Silva *et al.*, 2005; present study) can be considered a new reproductive strategy within the genus. Among the five reproductive modes recognized for the genus *Leptodactylus* by Prado *et al.* (2002), the reproductive mode of *L. labyrinthicus* herein described, with foam nests in excavated basins close to water, initial larval nourishment on trophic eggs, and late larval phase in the water, may be considered as an intermediate step between the mode described for *L. podicipinus*, with foam nests placed in open excavated basins close to water, and that of *L. fallax*, with foam nests placed in burrows far from the water and female provisioning of trophic eggs, allowing whole larval development inside the nest. As suggested previously (Prado *et al.*, 2002; Rodrigues Silva *et al.*, 2005), this strategy may be widespread among species in the *L. pentadactylus* group. The evolution of terrestrial reproductive modes in the genus *Leptodactylus*, with the deposition of eggs in places far from the water (Prado *et al.*, 2002), was apparently accompanied by the evolution of terrestrial modes of larval nourishment based on trophic eggs.

Leptodactylus labyrinthicus tadpoles and metamorphs that were fed on trophic eggs and subsequently on dry fish food reached larger sizes than those fed only on dry fish food. This suggests that the ingestion of trophic eggs is an adaptation to improve tadpole growth and not a fortuitous feature. Our results seem to support the hypothesis that the deposition of trophic eggs is important for the maintenance of the tadpoles, which can eventually reach metamorphosis by feeding only on these eggs (Rodrigues Silva *et al.*, 2005; present study). In natural conditions, it may be advantageous for the tadpoles to remain in the foam nest feeding on nutritive trophic eggs instead of going to the water to feed, since this implies predatory risks and energy expenditure related to prey search and capture. Furthermore, since *L. labyrinthicus* occurs in environments with unpredictable rainfall (Agostinho, 1994; Eterovick & Sazima, 2000b), tadpoles may perhaps be able to remain in the foam nest for a longer period of time until the next rainfall. However, alternative explanations could be responsible for the differences in the size of the metamorphs. It is possible that dry fish food is less nutritive than the trophic eggs or, as tadpoles fed on dry fish food were reared in the water without foam, we can not exclude the possibility that the higher growth rate of tadpoles may have been influenced by some chemical compound in the foam that promotes faster growth (e.g., growth hormone). Moreover, foam nests were also consumed by tadpoles reared in Treatments I and III. Contrary to this idea, it has been suggested that some biological properties of the natural foam or the foam made by the tadpoles, inhibit growth of tadpoles (Pisano & Del Rio, 1968; Downie, 1994). Although these stud-

ies were made with *Leptodactylus* species belonging to the *L. fuscus* group (Heyer, 1969), *L. labyrinthicus* tadpoles are also capable of making foam (W. R. Rodrigues Silva & A. A. Giaretta, pers. comm.). Additional experiments are necessary to study the effects of foam on *Leptodactylus* tadpole growth and survivorship.

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THE ROLE OF RELIEF IN LOCAL ABUNDANCE PATTERNS OF THE SPUR-THIGHED TORTOISE *TESTUDO GRAECA GRAECA* IN SOUTH-EAST SPAIN

J. D. ANADÓN¹, A. GIMÉNEZ¹, I. PÉREZ², M. MARTÍNEZ² AND M. A. ESTEVE²

¹Área de Ecología. Dpto. de Biología Aplicada, Universidad Miguel Hernández, Campus de Elche, Alicante, Spain

²Dpto. de Ecología e Hidrología, Universidad de Murcia, Murcia, Spain

We searched for patterns in the local abundance of *Testudo graeca graeca* with respect to relief characteristics in south-east Spain. The detection of tortoises during linear transects, in sampling plots and through fortuitous observations was used to examine distribution in relation to aspect and to topographic position at two different local scales. Both aspect and run-off gradients produced spatial patterns in the local abundance of *T. g. graeca*. These patterns are discussed with regard to the key conditions and resources for the species (solar radiation, food and nesting sites). At both scales tortoises were more abundant on north-west facing slopes, where solar radiation levels are assumed to be moderate to high, and where there are probably plenty of food resources. The apparent avoidance of north-east facing slopes, despite an abundance of food, suggests that tortoises may not be able to thermoregulate adequately in these areas and that thermoregulation may be the most important requirement for the species. Valley bottoms, mainly occupied by non-irrigated crops, were also selected. Therefore, our results suggest that there is a spatially aggregated population structure at a local scale that is caused by factors associated with relief.

Key words: aspect gradient, Chelonia, ecology, run-off gradient, topography

INTRODUCTION

The spur-thighed tortoise *Testudo graeca graeca* L. 1758 is a medium-sized terrestrial tortoise that mainly inhabits semi-arid environments. It has a westerly circum-Mediterranean distribution and the largest European population is spread over ca. 3000 km² in south-east Spain. In this area, the environmental factors that explain its distribution at a regional scale here are well known (Anadón *et al.*, in press), *T. g. graeca* occurs in semi-arid shrub habitats linked to mountain systems and is absent from large plains and basins. In the region of Murcia, which encompasses 60% of the range of the species in southeast Spain, there are 17 different geographical population units, ranging from 1 to 300 km², which are mainly associated with mountain systems and their piedmonts (Giménez *et al.*, 2001). However, very little is known about the spatial ecology of the species at a local scale.

During this study we searched for patterns in the local abundance of the spur-thighed tortoise with regard to the principal gradient types - topographic (runoff) and aspect - associated with relief. The topographic gradient is determined by the existence of an exporter zone (at the top) and an importer zone (at the bottom), between which there are important spatial differences in soil composition and moisture and, as a result, in vegetation and productivity (Forman & Godron, 1986). Different compass orientations give different levels of solar radiation and, therefore, microclimatic variations related to temperature and moisture levels occur, which can have

important consequences on both vegetation and productivity. Aspect is the variable that best explains variations in vegetation composition and soil moisture at a local scale in the semi-arid shrublands present in the south-east of the Iberian peninsula (Tong, 1989; Ferrer Castán, 1994). Terrestrial tortoises are ectothermic herbivores and have limited mobility; they may therefore show pronounced spatial patterns caused by environmental heterogeneity associated with local relief features. Their thermoregulatory and trophic strategies may promote habitat selection processes, which thus create spatial patterns in habitat use and local abundance.

MATERIALS AND METHODS

STUDY AREA

Our study was carried out in the Biological Reserve "Las Cumbres de la Galera" in the Sierra de la Carrasquilla (Murcia, Spain), managed by the Foundation 2001 Global Nature. It has a mean annual rainfall of 295 mm and a mean annual temperature of between 18-19°C (Ramírez, 1990). The altitudinal range of the reserve is 515-655 m a.s.l and it is characterised by a system of valleys (ramblas) with hillsides of moderate-to-steep gradients. Inside the reserve (70 ha), we established a study area of 29.7 ha that included three slopes facing in different directions belonging to two perpendicular watersheds (Fig. 1). The vegetation present in the study area includes *Anthyllis cytisoides* shrubland, patches of *Stipa tenacissima* and mixed shrubland of mainly *Rosmarinus officinalis*, *Artemisia* ssp., *Thymus* spp. and *Cistus* spp. The *Anthyllis cytisoides* shrubland is a secondary growth community that is usually caused by the abandonment of marginal

Correspondence: J. D. Anadón Herrera, Área de Ecología, Universidad Miguel Hernández, Edif. La Galia, Campus de Elche, Elche 03202, Alicante, Spain. E-mail: jdanadon@umh.es

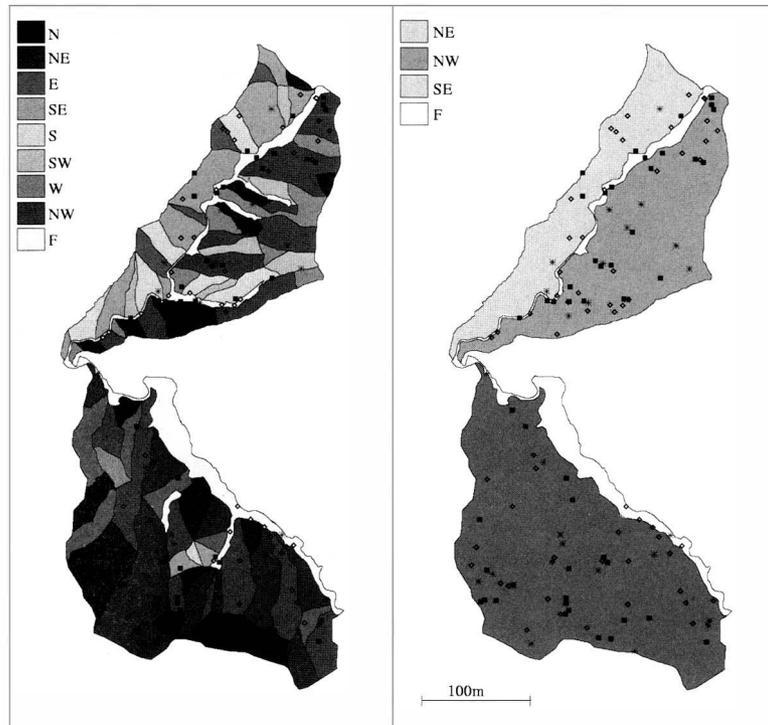


FIG 1. Orientation of slopes in the study area at large (left) and small scales (right) and locations of tortoises (squares = males, diamonds = females, asterisks = immatures).

farmland. In fact, part of the study area was cultivated until 1960, and there are still some small fields of non-irrigated crops in the bottom of the ramblas. The area is considered to be optimum habitat for the species according to habitat quality models developed at a regional scale (Giménez *et al.*, 2001). Capture-recapture studies in the study area have estimated a density of 16 tortoises/ha (authors, unpublished data). This value is high when compared with other *T. g. graeca* populations (Andreu *et al.*, 2000; Slimani *et al.*, 2002).

RELIEF CHARACTERISATION

We mapped aspect and topographic position with a GIS (GRASS 5.0, <http://grass.itc.it/>). We used water-

TABLE 1. Number of patches (*n*), percentage of the study area occupied by each one of the classes of the compass orientation variables, and number of tortoises located (NTOR), at the large and small scale.

Classes	Large scale			Small scale		
	<i>n</i>	%	NTOR	<i>n</i>	%	NTOR
N	-	-	-	14	16	27
NE	1	50.42	59	10	17.35	14
E	-	-	-	10	8.19	6
SE	1	26.39	15	10	9.72	11
S	-	-	-	7	5.7	5
SW	-	-	-	6	3.65	1
W	-	-	-	9	5.67	8
NW	1	15.25	49	17	24.01	41
Flat relief (F)	1	7.94	13	2	9.71	23

sheds as mapping references at two different scales. In species-habitat relationships, different scales of observation may lead to different patterns and processes (Wiens *et al.*, 1987; Wiens, 1989; Turner, 1989) and the detection of a given pattern will depend, partly, on the use of an appropriate scale. For this reason, we employed two different scales of observation. Third-order watersheds were used as reference for large-scale mapping, while first-, second- and third-order watersheds were used as references for small-scale mapping. The large scale reflected the main slopes of the study area, while the small scale reflected the different more minor slopes, which form part of the major slopes. The use of watersheds as references for scale mapping is an objective criterion for defining boundaries and yields two scales, which are strongly related to the natural organisation of landscapes.

Aspect was described by two variables linked to the two different scales (Fig. 1 and Table 1). Within these two variables there were nine possible classes corresponding to the points of the compass: N, NE, E, SE, S, SW, W, NW and F (flat relief). Four classes only were present at the large scale (SE, NW, NE and F), each one linked to one major slope plus the valley bottoms (F). The small scale (small slopes) exhibited all nine classes. Mean patch size for the slopes (valley bottoms included) at the large scale was 7.43 ha, while mean patch size for the slopes at the small scale was 0.35 ha.

In order to describe the topographic position on each slope we divided slopes at both large and small scales into three parts (Fig. 2): bottom (gully), middle (slope) and top (ridge). At the small scale, the top was defined as a buffer zone of 10 m wide around the first-, second-

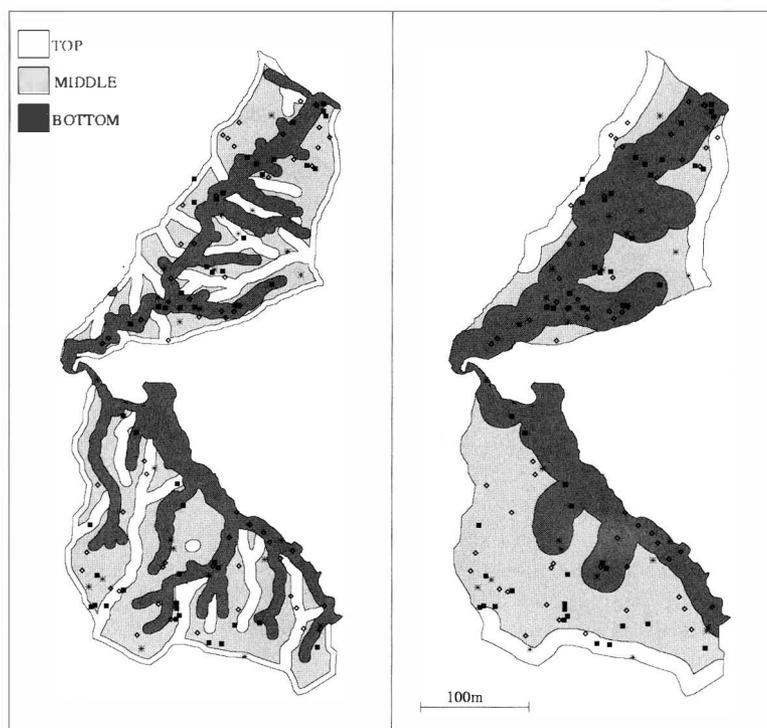


FIG 2. Parts of the slope in relation to the topographic (run-off) gradient in the study area at large (left) and small scales (right) and locations of tortoises (squares = males, diamonds = females, asterisks = immatures).

and third-order watersheds. The bottom was defined as a buffer zone of 10 m wide around the valley bottom (first-, second- and third-order valleys). The middle sector was defined as being that part of the slope that was not contained either within the top or the bottom sectors. At the large scale, linked to the major slopes, we constructed three variables using a buffer-zone width of 20, 30 and 40 m respectively.

SURVEYING METHODS

For the habitat selection analysis, we used the detection of tortoises during linear transects, surveys in sectors and fortuitous observations from other studies in the area. The area was intensively surveyed from the end of February to June, and in September and October, in hours of maximum activity for the species (at midday or in the morning, depending on the period of the year) (Andreu *et al.* 2000; Pérez *et al.* 2002). For sampling by sectors, we divided the study area into twelve sectors of equal area. All the sectors were surveyed simultaneously for two hours by three observers per sector. This sampling methodology was carried out eight times between 1997 and 2000. In 1997 we carried out nine linear transects, covering the study area evenly. The third group of tortoise locations were fortuitous sightings arising from vegetation sampling campaigns and surveys of tortoises for other purposes (Anadón, 2002). We recorded most locations of tortoises with a GPS and if a GPS was not available, sightings were located on aerial photographs (scale 1:5000).

Individuals were classified as males, females or immature. We considered immature animals to be less than seven years old (Andreu *et al.*, 2000). We used the

number of scute rings as an age estimator (Castanet & Cheylan, 1979; Germano & Bury, 1998). Males and females were distinguished visually since clear morphological differences exist (Andreu & López-Jurado, 1998).

DATA ANALYSIS

We compared the observed distribution of tortoises with a random distribution of variables related to aspect and topographic position using a χ^2 test. Recaptures were not taken into account during the analyses. If there were significant differences between classes ($P < 0.05$), an analysis of subdivided contingency tables was performed in order to assess the classes that were responsible for the differences (Zar, 1984). In this second analysis, all pairwise differences between adjacent classes were tested by searching for the groups of classes with minimal intra-group differences and maximal differences between groups, as expressed by their two values. In the variables related to the aspect gradient, the class referring to flat areas (F) was not included in any group and was considered in itself as a separate group since it does not belong to the circular aspect gradient. Analyses of the variables with regard to aspect at the small scale were made for the whole study area and for each major slope (orientation at the large scale) separately in order to detect whether the preferences for orientation at the small scale (small slopes) also held for the differing major slopes. These analyses were made for all individuals and for males and females separately. We also performed a previous test comparing the distribution of males and females in relation to each one of the environmental variables.

TABLE 2. Relevant results of the χ^2 test and partial χ^2 test for compass orientations at the large scale.

Classes	df	χ^2	<i>P</i>
NE, NW, SE and F	3	8.16	0.0429
NE, SE	1	0.36	0.5479
(NE+SE), NW and F	2	7.317	0.0258

RESULTS

ASPECT GRADIENT

A total of 136 locations - 47 males, 61 females and 28 immatures - of different tortoises were made (Fig 1). The number of locations of tortoises per unit area varied substantially from one major slope to another (Table 1). Locations per unit area on the major north-west facing slopes were twice as frequent as those on the major southeast-facing slopes. There was a significant difference in the distribution of tortoises among aspect classes (Table 2). Analysis of subdivided contingency tables showed that tortoises were more abundant in the north-west facing slopes in comparison with flat areas (valley bottoms) and the group made up of north-east and south-east facing slopes (Table 2). This variable did not give any differences for males as opposed to females. At the small scale, there were also significant differences between classes (Table 3). Three homogeneous groups arose from the analysis of subdivided contingency tables: one group consisted of west, north-west and north facing slopes; another of north-east, east, south-east, south and south-west facing slopes and a third was made up of flat areas (hereafter, NW group, SE group and F). The SE group showed the least number of locations of tortoises. There were no significant differences between the NW group and F, whereas there was a significant difference between F and all the other compass orientations grouped together (Table 3). The resulting abundance ranking is: F > NW group > SE group.

More tortoise locations were found in the NW group than in the SE group when we compared these two groups separately within the NW and NE major slopes. No analysis was carried out on the SE major slope group since the NW group was not represented.

Finally, χ^2 tests did not detect differences in the distribution of males and females in relation to aspect, neither at the large nor at the small scale.

TABLE 3. Relevant results of the χ^2 test and partial χ^2 test for compass orientation at the small scale.

Classes	df	χ^2	<i>P</i>
N, NE, E, SE, S, SW, W, NW and F	8	21.5	0.006
(N+NW+W), (NE+E+SE+S+SW) and F	2	19.6	0.000
N, NW and W	2	0.2	0.882
NE, E, SE, S and SW	4	2.5	0.636
(N+NW+W) and F	1	2.2	0.135
(N+NW+W) and (NE+E+SE+S+SW)	1	12.5	0.000
(NE+E+SE+S+SW) and F	1	17.1	0.000

TABLE 4. Results of the χ^2 test of variables referring to 'position on the slope at small and large scales. The variable at the large scale is the one with buffer-zone width of 20 m, since it is the only one that yielded significant differences among its classes. T=Top part, M= middle, B=bottom.

	Large scale			Small scale		
	df	χ^2	<i>P</i>	df	χ^2	<i>P</i>
T, B and M	2	6	0.050	2	5.98	0.050
T and B	1	5.0	0.026	1	5.89	0.015
(T+B) and M	1	0.6	0.446	1	0.42	0.516
T and M	1	1.9	0.163	1	3.69	0.055
(T+M) and B	1	4.2	0.039	1	2.66	0.103
B and M	1	3.0	0.083	1	0.4	0.527
(B+M) and T	1	2.8	0.091	1	7.43	0.006

TOPOGRAPHIC POSITION ON THE SLOPE

For the topographic position at the large scale and for all individuals there were significant differences among the top, the middle and the bottom parts when the buffer-zone width was 20 m (Table 4), but not when the buffer-zone width was 30 or 40 m. In the first case, there was a significantly greater abundance of locations of tortoises at the bottom of slopes (Table 4). In this case, the resulting ranking comes out as: bottom > middle > top; and: bottom > (middle + top).

At the small scale we found a similar pattern (Table 4), although fewer tortoises were detected at the top of slopes. The resulting ranking is: bottom > middle > top, and: (bottom + middle) > top.

Analyses for males and females did not show any differences in their abundance at either large or small scale.

DISCUSSION

ASPECT GRADIENT

In our study area, during the activity seasons (spring and autumn), spur-thighed tortoises were most abundant in north-westerly orientations at both local scales. To fully understand this pattern we should attempt to understand the differential habitat characteristics present in slopes of different aspects. Two key conditions/resources for the species - solar insolation and food - are expected to vary along this gradient as a result of microclimatic differences (Noy-Meir, 1973).

Solar radiation is highest on south facing slopes and lowest on north facing slopes. This radiation gradient leads to gradients in ground moisture and vegetation in the south-east of the Iberian peninsula with maxima in NNE facing and minima in SSE facing mountain systems (Ferrer Castán, 1994). This lack of complete coincidence between the solar and the moisture/vegetation gradients is due to the greater dryness of western orientations (evening insolation) as opposed to eastern orientations (morning insolation) (Dargie, 1984, 1987). With regard to food resources, *T. g. graeca* is a generalist herbivore that mainly feeds on herbaceous plants. In the other Iberian populations of the species, analyses of faecal pellets revealed 88 plant species belonging to 26 families (Andreu *et al.*, 2000). Annual

grass cover could be thus useful as an approximate descriptor of food availability. In that sense, the analysis of previous habitat data from the reserve (Anadón, 2002) showed that grass cover in the study area was greatest in the north-west, closely followed by the west, north and north-east, whereas minimum values were found in the south and south-east.

Thus, slopes where tortoises were more abundant (north-west facing) are assumed to have low-to-medium radiation rates and moderate to high levels of food resources. However, not all areas with high levels of food resources had equal tortoise abundance values. North-east facing slopes were rejected despite their abundance of annual grasses. It may be that these slopes, which have the shadiest aspect (Ferrer Castán, 1994), were too humid and cold for the species. In this case, thermoregulation could be the most important factor influencing abundance and habitat-use, and could even condition food-finding strategies. This agrees with the general patterns of habitat selection in ectotherms in temperate climates (Grant, 1990; Reinert, 1993). On the other hand, low abundance values of tortoises on south-facing slopes could be due to both lack of food resources, since these aspects presents the lowest values of annual grass cover, and thermoregulation. Thermoregulatory behaviour of *T. g. graeca*, as in most ectotherms in temperate climates, relies on a balance between the warming and cooling of their bodies, by both basking in the sun and resting in shadier and cooler areas (Lambert, 1981; Meek & Jayes, 1982; Sturbaum, 1982). South-facing slopes could be too hot for the species and may lack a proper combination of sunny and cool areas. Anyway, further field work and experimental studies are needed to fully address the thermoregulatory requirements of the species in relation to radiation rates and vegetation cover, and the specific distribution of food resources.

Aspect may give rise to areas with different habitat quality for the species and different tortoise densities, yielding an aggregated distribution pattern at a local scale. These results agree with those found by Andersen *et al.* (2000) on *Gopherus agassizii* in a location in the Mojave desert in which the variable that best explained habitat suitability for the species (defined in terms of the abundance of tortoises) was aspect. In relation to scale, the same abundance patterns related to aspect have emerged on the two scales we have employed. It suggests on one hand that the processes operating on these two scales are likely to be the same (Wiens, 1989) and, on the other, that the two scales may be useful in order to detect local abundance patterns.

Finally, it should be pointed out that the detected abundance pattern related to aspect is specific for the study area and it is expected to vary throughout the distribution range of the species wherever there is a climate gradient conditioning thermoregulatory strategies and the distribution of food resources. Moreover, under the same climatic conditions, differences in relief structure, usually associated with different lithologies, or different

successional stages in the vegetation, may lead to different landscape patterns and, thus, to different selection strategies and spatial patterns of abundance (Turner, 1989).

RUNOFF GRADIENT

Tortoises were most abundant in flat areas located in valley bottoms. Valley bottoms, mostly occupied by small non-irrigated crops, could be used for egg-laying, as has been observed in other *Testudo* populations (Swingland & Stubbs, 1985). On the other hand, crops in valley bottoms have a very high perimeter-area ratio (see Figs 1 and 2) and could be considered as edge-habitats between crops and shrubs. In this sense, edges have been identified as a favourite habitat in other reptiles (Blouin-Demers & Weatherhead, 2001, 2002).

The high frequency of tortoise locations in crops agrees with the positive effect at a local scale of open areas derived from human activities, as has been found in other species of tortoises in temperate regions (Auffenberg & Franz, 1982; Diemer, 1986; Kazmaier *et al.*, 2001). The use of crops by tortoises points towards future directions in research into the relation between the effects of traditional anthropic fragmentation and the use of edges by these species, a line of investigation that may provide useful insights into a number of conservation issues.

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SHORT NOTES

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AN EXAMINATION OF *PIPA PARVA* (ANURA: PIPIDAE) FROM NATIVE AND INVASIVE POPULATIONS IN VENEZUELA

G. JOHN MEASEY¹ AND RAMIRO ROYERO²¹Zoology Department, The Natural History Museum,
London, UK²Fundacion para el Desarrollo de las Ciencias Fisicas,
Matemáticas y Naturales (FUDECI), Caracas, Venezuela

Invasive populations of anurans contribute to global amphibian declines, and many instances involve pipid frogs. Here we report on an invasive population of *Pipa parva* in Carabobo State, and a native population in Zulia State, Venezuela. The frogs were found in high densities in a fish farm near Lake Valencia, and had a diet of benthic invertebrates. Invasive *P. parva* were large compared to those described in previous reports (mean snout-vent length: 37.34 ± 0.73 mm), but the native population was found to be significantly longer (mean snout-vent length: 44.08 ± 1.34 mm). Colonisation by terrestrial movement and potential impact of this invasive species are discussed.

Key words: diet, invasive amphibians, morphology, South America

While much recent attention has focussed on amphibian declines (Houlahan *et al.*, 2000), the incidents of problematic introduced amphibians continue to rise (Kats & Ferrer, 2003). Thorough investigations of some invasive anurans have exemplified a wide range of mechanisms of impact, including: competition, disease, toxicity and predation (e.g. Cunningham & Langton, 1997; Kupferberg, 1997; Lafferty & Page, 1997; Crossland, 2000). Results suggest a positive relationship between density and impact, and in some studies, that high densities are a significant factor in concerns over global amphibian decline.

The family Pipidae has five extant genera of principally aquatic anurans, four (*Hymenochirus*, *Pseudhymenochirus*, *Silurana* and *Xenopus* in sub-Saharan Africa and one (*Pipa*) in central and South America. The best known invasive pipid is *Xenopus laevis*, and its detrimental effects have been documented in California, UK and Chile. For example, an endangered fish (*Eucyclogobius newberryi*) was found in the gut contents of *Xenopus laevis* inhabiting the estuary of the Santa Clara River, California (Lafferty & Page, 1997), and in both UK and Chile *X. laevis* probably con-

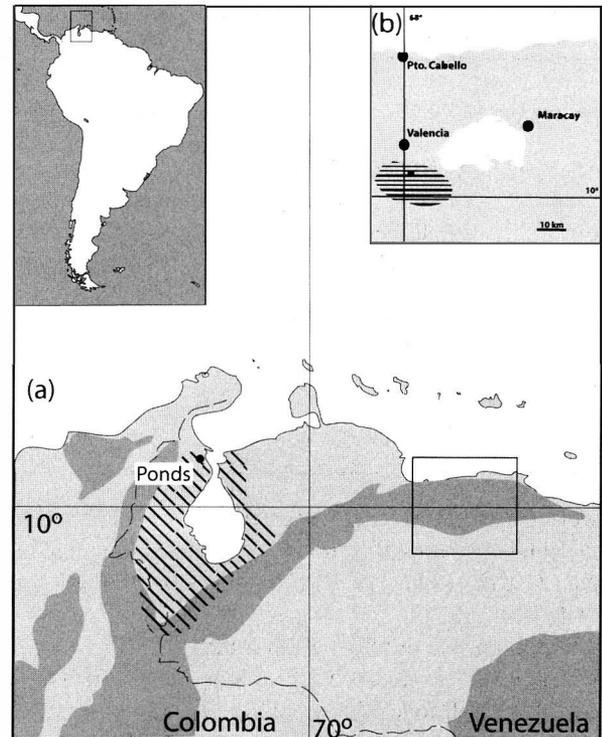


FIG. 1. Known distribution of *Pipa parva* in Venezuela and Colombia (right sloping lines) with the extension of the Cordillera Oriental shown as shaded areas (a) The range is approximated with data from Trueb & Cannatella (1986), Gines (1958) and Barrio & Fuentes (2000). (b) The introduced range (horizontal lines) is approximated from the area, south of Valencia, where fish farmers have reported *P. parva* (personal communication to RR). The location of Ventuari fish farm is shown with a black square.

sumes local amphibian eggs and larvae, as well as causing trophic cascades by consumption of benthic macroinvertebrates (Measey, 1998a; Lobos & Measey, 2002).

The genus *Pipa* is represented by four species in Venezuela (Gines, 1958): *Pipa aspera*, *P. parva*, *P. Pipa* and *P. arrabali*. *Pipa parva* is endemic to the Lake Maracaibo region (Zulia and Tachira States) in the north east of Venezuela (Trueb & Cannatella, 1986). Lake Maracaibo lies in the north-west corner of Venezuela bordering Colombia, and *P. parva* is restricted in its distribution by mountains to the west (Perija Range) and east (Cordillera de Merida), both extensions of the Cordillera Oriental, which dominates the region (Fig. 1). Royero & Hernandez (1996) reported the presence of *Pipa parva*, outside of its native Lake Maracaibo drainage, in the western area of Lake Valencia, Venezuela (Fig. 1). The movement of *P. parva* to Carabobo State is attributed to anthropogenic means, with the intention of breeding individuals for the national and international market in ornamental aquarium livestock.

Here we examine an invasive population of *Pipa parva*, collected from a fish farm in the Valencia area (Carabobo State), and compare them to a sample of animals from the native Maracaibo drainage (450 km

Correspondence: G. J. Measey, Laboratoire d'Ecologie des Sols Tropicaux (UMR 137), Institut de Recherche pour le Développement, 32 Avenue Henri Varagnat, 93143 Bondy Cedex, France. E-mail: john@measey.com

TABLE 1. Stomach contents of 42 *Pipa parva* from an invasive population caught at the Ventuari fish farm, near Valencia, and five *P. parva* caught in a pond from a native population near Maracaibo, Venezuela.

	Total frequency	% occurrence	Average frequency	Total mass	Average mass	Mass / frequency
VENTUARI FISH FARM, VALENCIA						
Chironomid larvae	899	93	21.4	1.057	0.025	0.001
Ephemeropteran larvae	31	33	0.7	0.109	0.003	0.004
Coleopteran larvae	34	33	0.8	0.147	0.004	0.004
Ostrocod (long)	64	21	1.5	0.017	0.000	0.000
Ostrocod (small)	46	24	1.1	0.009	0.000	0.000
Snail eggs	1	2	1.3	0.044	0.001	0.044
MARACAIBO, VENEZUELA						
Planorbid snails	7	40	3.5	0.3851	0.077	0.55

west in Zulia State). Particular attention is paid to one of the chief attributes of an invasive species: individual impact (Parker *et al.*, 1999) through the examination of diet.

Ventuari fish farm lies 8 km south of Valencia on the Valencia-Las Lomas road, Carabobo State (10° 06' N; 68° 08' W. Altitude 520 m asl; Fig. 1b). The farm has 20 outdoor holding ponds (approx. 10 × 50 m and 1 m depth), nine of which are within an anti-bird mesh enclosure, and all ponds are bounded by a 0.5 m anti-crocodile wall. *Pipa parva* were collected using unbaited funnel traps with 50 mm diameter openings. Traps were regularly left submerged in each pond throughout the day and emptied in the evenings when most of the catch would normally have drowned (E. Garcia, pers. comm.). Pools were occasionally drained and sun dried as a means of reducing the numbers of *P. parva*, as well as liming with calcium hydroxide to reduce the risk of disease, parasites and fungal infections to farmed fish.

On the 3 April 1997, the live contents of traps from six meshed holding ponds were pooled in a glass

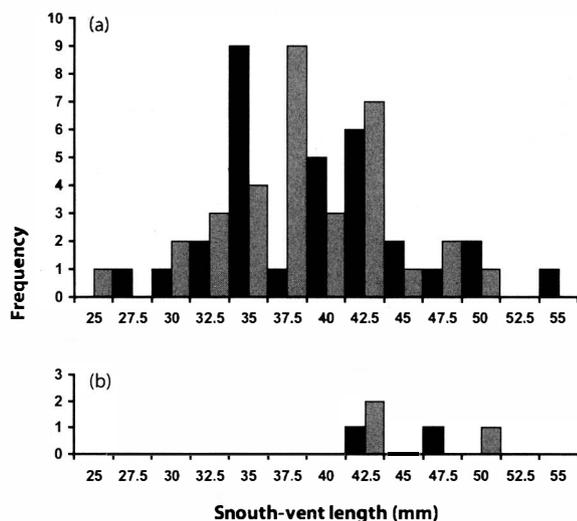


FIG. 2. Frequency distribution histograms for snout-vent length of *Pipa parva* caught (a) from an invasive population in Ventuari fish farm, south of Valencia, and (b) in a native population north of Maracaibo, Venezuela. Black bars are females and grey shaded bars males.

aquarium tank overnight. The following day, the contents of the same traps were removed live at 1500 hr. Water temperature was 28.0° C and the mud substrate 27.4° C. The pH of the water was 10.54; this elevated level presumably due to liming.

Ponds and irrigation ditches were sampled, using seine and pond nets, along the highway to Dami Tulé, Zulia State (10° 43' N; 71° 43' W. Altitude 15 m asl; Fig. 1a), and within the native range of *Pipa parva*. *P. parva* were only found in one small pond (approx. 5 × 6 m and 1.5 m depth), which was repeatedly seined. Temperature and pH of the water were noted as 32.9° C and 9.37 respectively.

After collection, specimens of *Pipa parva* were lethally anaesthetised with MS222 (Sandoz) and preserved with 10% formalin, which was also injected into the body cavity. Specimens are to be deposited into the collection of Natural History Museum, London (BMNH). Snout-vent length (SVL) measurements were made using dial callipers to the nearest 0.1 mm. Stomach contents were removed and identified using a binocular dissecting microscope. Sex was determined from direct examination of gonads during dissections. A two-way ANOVA (Statistica v. 5.5A, StatSoft, France) was used on log (ln) normalised SVL data to test for differences between sexes and populations.

A total of 64 *Pipa parva* were collected and preserved for use in this study. Five animals (three males, two females) were obtained using seine nets from the pond near Lake Maracaibo (Fig. 2b). More animals were seen to escape the seine net as it was retrieved, indicating that this was not the total population of the pond. The six traps collected at the fish farm yielded 42 *P. parva* on 3 April, 1997, with an extra 17 animals captured the previous night (total of 59: 30 males, 29 females; Fig. 2a). All 64 animals were used in the morphological analysis, but the 17 animals kept overnight were not included in the analysis of diet.

Female *P. parva* from the fish farm were found to be slightly longer (mean±SD = 37.91±1.18 mm) than males (mean±SD = 36.77±0.86 mm), although this difference was not significant (two-way ANOVA: $F_{1,60} = 0.029$; $P=0.865$; Fig. 2). Despite the small sample size,

the native population had larger mean SVL for females (mean±SD = 44.0±2.00 mm) and males (mean±SD = 44.13±2.15 mm), and these animals were found to be significantly larger than specimens from the fish farm (two-way ANOVA: $F_{1,60} = 6.32$; $P=0.015$; Fig. 2). The interaction between sex and site was not significant (two-way ANOVA: $F_{1,60} = 0.038$; $P=0.846$).

Table 1 shows the composition of stomach contents of animals captured at the fish farm, all with items of benthic origin. Most individuals (93%) had ingested chironomid larvae, which made up the major component of stomach contents in both frequency and mass. Other items also included aquatic insect larvae (Ephemeroptera and Coleoptera), as well as two species of ostracods. One individual had ingested snail eggs. Only two stomachs had contents from animals captured near Maracaibo, both of these contained aquatic snails (Table 1).

Despite the small sample size, *Pipa parva* from the natural population in Maracaibo were found to have a significantly greater SVL than the invasive population from the fish farm in Valencia. Trueb & Cannatella (1986) report that SVL of museum specimens of *P. parva* they studied are smaller than those found here: means for females are given as 33.6±1.2 mm (female SVL range 27.0–44.3 mm; $n=19$), and for males as 31.7±0.5 mm (male SVL range 27.9–37.0 mm; $n=21$). Trueb & Massemin (2001) report on the sizes of a small sample of *Pipa aspera* which had a significant size dimorphism between sexes (two tailed *t*-test on data from their Table 1), but no significant sexual size dimorphism was found for *P. parva* despite the larger sample size.

Trueb & Massemin (2001) claim that *P. parva* is the smallest of the 'micropipas', but we show here that different populations can have different mean sizes. Fig. 2a shows larger maximum sizes for both females (SVL 55.0 mm) and males (SVL 46.1 mm), both from the Ventuari fish farm. That animals from the Maracaibo population were significantly larger suggests that SVL ranges may be even greater than those reported here. Trueb & Cannatella (1986:438) also report that the 'venter of the body and limbs is grayish tan, and either immaculate or bearing a few, small, indistinct spots in the pectoral region of some individuals.' We found that all animals collected (both from Maracaibo and Ventuari fish farm) had lightly spotted venters, which did not fade in preservation.

Our results suggest that *P. parva* is a generalist predator of appropriately sized aquatic fauna. Gines (1958) describes the diet of Venezuelan frogs of the genus *Pipa* as consisting of larval insects as well as small fish and tadpoles. Similar prey was reported to be taken by captive *P. aspera*, including tadpoles of *Hyla boans* and minute characid fish (Trueb & Massemin, 2001). *Pipa arrabali* was found to exert strong predatory pressure and influence the distribution of *Osteocephalus taurinus* eggs and tadpoles (Gascon, 1992). Buchacher (1993) found that *P. arrabali* were quick to devour tad-

poles of *Phyllomedusa bicolor* which dropped into a pond from a foam nest. He also observed that *P. arrabali* left their ponds to prey upon larvae of *Leptodactylus knudseni* in terrestrial foam nests. Another pipid, *Xenopus laevis* was also found to take many items of terrestrial origin, presumed to have fallen into the surface film, but also being snatched from around the water body (Measey, 1998a). However, for ingestion it is necessary for *X. laevis* to return to the water (Measey, 1998b), and this appears not to be the case for *Pipa* (see Buchacher, 1993). Another notable difference between the diets of frogs from these two genera is the absence of planktonic prey items reported from the diet of *Pipa*. This may simply reflect the abundance of available benthic prey, although Measey (1998a) showed that most *X. laevis* still took planktonic prey whilst benthic prey was not only available, but abundant.

Royero & Hernandez (1996) speculated on an economic impact by *P. parva* on fish farmers. While our results do not find that these frogs prey on either fish or fish fry, our sampling was not exhaustive, and the extent of predation and/or competition with farmed fish lies open to further investigation. That *P. parva* are predators of farmed fish and fish fry, or that they are in competition with farmed fish by eating fish food, as claimed by Royero & Hernandez (1996), seems likely. Outside of the fish farms, it is probable that *P. parva* are also predators of other native Venezuelan anuran eggs and larvae, as well as fish. Most reports of the effects of invasive amphibians have an emphasis on native amphibian fauna and highlight direct predation on amphibians by adult invaders, although this is likely to be an artefact of the types of studies conducted. Few studies have considered predation pressure on native invertebrate fauna. However, assessments of total impact (area occupied, abundance, and impact per individual sensu Parker *et al.*, 1999) are absent from the literature, and yet desperately needed.

The fish farmers of Valencia claimed that during the onset of big rains, the farms are invaded by thousands of *Pipa parva*, and this requires the frogs to climb over the 0.5 m anti-crocodile walls surrounding the farm. In addition, Royero & Hernandez (1996) stated that fifteen days after constructing new pools at a fish farm near Valencia, these were found to contain a large number of *P. parva* larvae which were thought to compete with fish for food.

Pipids are often thought of as completely aquatic frogs which rarely venture out of the water. Péfaur & Cardoso (1992) made notes on *Pipa carvalhoi* and *P. parva* walking and jumping out of water. Péfaur observed *P. parva* jump in a very cumbersome manner, reaching a height of 0.7 m but moving horizontally only half of this distance (Péfaur & Cardoso, 1992). This ability to move overland may explain the rapid invasive process over a large portion of the Valencia area. Interestingly, it appears that *Pipa carvalhoi* also occurs in invasive populations in Rio State, Brazil (E. Dubauskas, pers. comm.).

Venezuela has legislation designed to protect against invasive species (resolution No. 260 of the Ministry of the Environment and Renewable Natural Resources), although Royero & Hernandez (1996) comment that there are not sufficient resources to tackle such problems. Urgent studies are needed in order to assess the natural and economic impact of *Pipa parva* in the vicinity of Valencia. Special efforts should be made outside of the fish farms to determine whether these frogs have entered into the Lake Valencia drainage system. Within fish farms, evidence is needed of predation or competition by both adults and larvae, as claimed by fish farmers, and a quantification of economic impact per individual. The use of unbaited funnel traps within fish ponds provides a practical and inexpensive manor of trapping unwanted *Pipa parva*. This method could be augmented by occasionally baiting the traps, as described by Measey & Tinsley (1998). In order to reduce invasions by *P. parva* moving overland, drift fencing and pit fall traps could be constructed behind the anti-crocodile wall. The enforcement of existing Venezuelan legislation is pivotal in reducing growing problems of invasive species.

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**SEASONAL CHANGES IN THE
THERMAL ENVIRONMENT DO NOT
AFFECT MICROHABITAT
SELECTION BY *PSAMMODROMUS
ALGIRUS* LIZARDS**

JOSÉ A. DÍAZ¹, SARA CABEZAS-DÍAZ^{1,2} AND
ALFREDO SALVADOR³

¹*Dpto. de Zoología y Antropología Física (Vertebrados),
Facultad de Biología, Universidad Complutense, Madrid,
Spain*

²*Instituto de Investigación en Recursos Cinegéticos
(IREC), C.S.I.C., Ciudad Real, Spain*

³*Dpto. de Ecología Evolutiva, Museo Nacional de
Ciencias Naturales, C.S.I.C., Madrid, Spain*

We studied the thermal consequences of microhabitat selection by *Psammmodromus algirus* lizards by combining data on the frequency of use and relative availability of three different types of microhabitats, with information about the environmental operative temperatures, and their deviations from the lizards' selected thermal range, available in these microhabitats. In both seasons, lizards preferred holm oak shrubs and avoided rockrose shrubs and open areas. However, the thermal suitability of holm oaks was highest in July but lowest in May. We suggest that microhabitat preferences were more related to other aspects of lizard ecology (e.g. antipredator behaviour) than to thermal requirements.

Key words: lizard, operative temperatures, predation risk, thermoregulation

It is well known that lizards actively select the microhabitats they use according to their type of substrate, elevation, slope, plant cover, and other structural features (Arnold, 1987; Carrascal *et al.*, 1989; Díaz & Carrascal, 1991). Changes in microhabitat selection may also counteract the temporal variation or spatial heterogeneity of the thermal environment (Porter *et al.*, 1973; Christian *et al.*, 1983; Adolph, 1990), and many studies have emphasized the thermoregulatory consequences of habitat selection by ectotherms (Christian *et al.*, 1983; Grant & Dunham, 1988; Adolph, 1990; Huey, 1991; Bauwens *et al.*, 1996; Gvodzik, 2002). In fact, the physiological consequences of habitat selection are of paramount importance for ectotherms because the environmental temperatures available vary spatially over time, and this variation translates into body temperatures that affect short-term physiological performance, thereby determining their long-term ability to grow, survive and reproduce (Huey, 1991).

Thermoregulatory responses to environmental seasonality have also been reported for a number of liz-

ard species (Van Damme *et al.*, 1987), sometimes including notable shifts in microhabitat use (Christian *et al.*, 1983; Schäuble & Grigg, 1998). However, if microhabitat selection is more dependent on other factors such as predation risk, social behaviour, etc., it may remain constant despite seasonal variations in the thermal suitability of microhabitats. Here, we report that *Psammmodromus algirus* lizards in a Mediterranean open forest do not modify their patterns of habitat selection despite significant seasonal variation in the thermal characteristics of different types of microhabitats.

Psammmodromus algirus is a medium-sized (adult snout-vent length ca. 60-90 mm), terrestrial, widely-foraging, lacertid lizard common in Mediterranean forests of the Iberian Peninsula. Our study was conducted at 'El Pardo' (Madrid, central Spain: 40°31'N, 03°47'W; 650-700 m elevation), a holm oak (*Quercus ilex*) broad-leaved, perennial forest whose shrub layer is dominated by holm oak offshoots and rockroses (*Cistus ladanifer*), alternating with open areas covered by annual herbs and/or bare ground. The undergrowth of holm oaks and rockroses forms well defined vegetation patches, with an average cover of more than two-thirds of the total surface. We distinguished three types of microhabitat: holm oak shrubs, rockrose shrubs, and open areas. The site has a mean annual temperature of 12.5 °C and a mean annual rainfall of 686 mm, but meteorological conditions vary widely among seasons (e.g. mean temperature and precipitation are 15.0 °C and 54.0 mm in May vs. 23.4 °C and 14.8 mm in July). Several predators, including birds (e.g. *Buteo buteo*, *Falco tinnunculus*, *Otus scops*, *Athene noctua*, *Upupa epops* and *Lanius excubitor*) and snakes (*Malpolon monspessulanus* and *Elaphe scalaris*) are common at the study site.

Field sampling took place in the spring (May) and summer (July) of the 1997 activity season. Data were collected on sunny days between 0700 and 2000 hr (Mean European Time), covering the whole daily activity period of *P. algirus*. In both seasons, we simultaneously collected data about operative temperatures (T_e s) and microhabitat selection. Data on operative temperatures were obtained using unpainted hollow copper cylinders (length=5 cm, diameter=1 cm) whose adequacy was experimentally confirmed (Walsberg & Wolf, 1996) following the procedures outlined by Belliure *et al.* (1996). In each season, copper models were distributed at random locations (1-9 m; distance determined by one-digit numbers from a table of random numbers) along the transects employed to measure the lizards' use of microhabitats. Temperatures were recorded at hourly intervals (final sample size of 1,170 T_e records per season); hourly variations in T_e were large enough to ensure that repeated measurements of the same model could be taken as independent data (Díaz & Cabezas-Díaz, 2004). When each model was placed on the ground for the first time, we also recorded its microhabitat location (holm oak

patch, rockrose patch, or open area, depending on the presence or absence of those shrub species within a 1 m-radius circle centered on the model location). The random distribution of models with respect to microhabitats provides the null hypotheses against which lizards' selectivity can be evaluated (Bauwens *et al.*, 1996; Díaz, 1997). Microhabitat use by lizards was determined by noting the location when first seen of all individuals sighted. We also noted the sex and size (adult or subadult; juveniles absent at this time of year) of all lizards seen. Because previous analyses showed that neither microhabitat use, thermoregulatory behaviour, nor body temperature vary significantly with the size or sex of the lizards (Díaz, 1997), all subsequent analyses are based upon pooled samples. In both seasons, the high population densities of *P. algirus*, combined with our effort to visit different areas each sampling day, reduced the probability of resampling individuals.

The selected temperature ranges (T_{sel}) that lizards attempt to maintain in the absence of physical and biotic constraints (Licht *et al.*, 1966) was estimated in the laboratory (Belluire *et al.*, 1996; Díaz & Cabezas-Díaz, 2004) using standard precautions. Thus, food and water were supplied *ad libitum*, the photothermal gradient employed offered a wide range of T_{cs} (23.4 - 51.7 °C), and body temperature was measured at different times of day on lizards of both sexes. Selected temperature ranges were estimated for each lizard as the central 80% of all body temperatures measured in the thermogradient (Gvodzik, 2002). The average T_{sel} increased ca. 2 °C from May (average lower and upper limits of 30.9 and 34.7 °C, respectively) to July (32.8-36.9 °C). Following Hertz *et al.* (1993), we evaluated the thermal suitability of microsites with reference to the T_{sel} of the corresponding season, using the mean of the absolute values of deviations of T_{cs} from T_{sel} (d_{cs}). Previous studies show that this species thermoregulates behaviourally in the field with great efficiency (Díaz, 1997; Díaz & Cabezas-Díaz, 2004).

The selection of microhabitats and its seasonal variation were analysed by means of a log-linear analysis (Heisey, 1985) of the contingency table of lizard and model observations, classified according to season and microhabitat type (Fig. 1). The final model obtained included the interactions season \times microhabitat (partial association: $G=12.41$, $df=2$, $P=0.002$) and model or lizard \times microhabitat ($G=44.37$, $df=2$, $P<0.001$); this is the simplest model that includes enough interactions to adequately explain the data, so that the observed and expected values do not differ significantly ($G=3.404$, $df=3$, $P=0.333$). The first interaction merely implies that the cover of holm oak shrubs at the sampled locations slightly decreased, and the cover of open areas slightly increased, from May to July. The second interaction is more relevant, because it shows that the number of lizards observed within or near the edge of holm oak shrubs was greater than expected (they chose shrub oaks

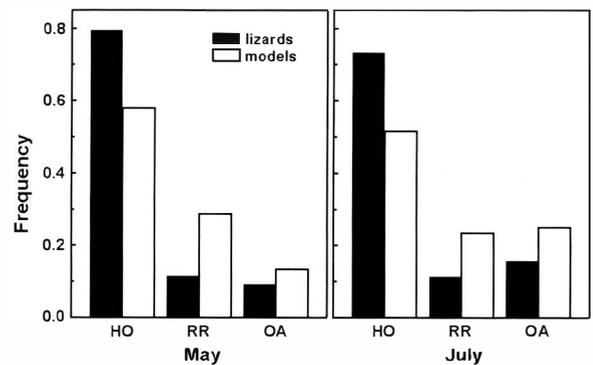


FIG. 1. Microhabitat selection by *P. algirus* in May and July: frequency of use (lizards, black bars) and relative availability (randomly distributed models, white bars) of three different types of microhabitats: holm oak shrubs (HO), rockrose shrubs (RR), and open areas (OA). Sample sizes are 341 in May and 340 in July for lizards, and 150 in May and 120 in July for models.

about 76% of the time, although this microhabitat accounted for about 56% of the available habitat), whereas the opposite was true for rockrose shrubs and open areas. Remarkably, the three-way interaction was not significant ($G=0.30$, $df=2$, $P=0.862$). Thus, lizards were selective in their use of microhabitats, but their preferences did not change seasonally.

We examined the thermoregulatory consequences of microhabitat selection using ANOVA contrasts to compare the T_{cs} and d_{cs} of holm oak shrubs (positively selected by lizards; contrast coefficient=2) against those of rockrose shrubs and open areas (avoided by lizards; contrast coefficients=-1). In both seasons, between-microhabitat differences were significant both for T_{cs} (May: $F_{1,1167}=12.4$, $P<0.001$; July: $F_{1,1167}=27.73$, $P<0.001$) and d_{cs} (May: $F_{1,1167}=6.90$, $P=0.009$; July: $F_{1,1167}=5.14$, $P=0.023$), and holm oak T_{cs} were always lower than rockrose or open areas T_{cs} (Table 1). Thus, the thermal suitability (i.e., mean d_{cs}) of the preferred microhabitat was highest in July but not in May, when most T_{cs} (76 %) were below T_{sel} . However, holm oak patches were actively selected in spring (Fig. 1) despite having the lowest thermal suitability in that season.

We also considered the percentage of operative temperatures in each type of microhabitat that fell below, within, and above T_{sel} (Table 2). These frequencies differed significantly among microhabitats both in May ($G=20.1$, $df=4$, $P<0.001$) and in July ($G=31.4$, $df=4$, $P<0.001$). In May, when most T_{cs} were too low, the proportion of models with T_{cs} below T_{sel} was highest in holm oaks and lowest in rockroses. In July, the proportion of T_{cs} above T_{sel} was highest in rockroses and lowest in holm oaks. However, in both seasons the proportion of models with temperatures within T_{sel} was low in all microhabitats (Table 1). This implies that lizards must be using other mechanisms of temperature regulation (such as the selection of sunlit or shaded patches, or shuttling between sun and shade; Díaz & Cabezas-Díaz, 2004) to thermoregulate effectively within each of the three microhabitat types, and that the contribution of

TABLE 1. Operative temperatures (T_e : mean \pm SD) and their deviations from the selected range (d_e : mean \pm SD), both in $^{\circ}$ C, of microhabitats classified as holm oak shrubs, rockrose shrubs, or open areas, depending on the presence or absence of those shrub species within a 1 m radius circle centered on the model location. N = sample size.

Microhabitat	May			July		
	T_e	d_e	N	T_e	d_e	N
Holm oak shrubs	24.9 \pm 9.2	8.6 \pm 6.0	716	33.3 \pm 12.6	7.8 \pm 8.0	646
Rockrose shrubs	27.4 \pm 10.0	7.6 \pm 6.1	286	37.4 \pm 13.0	8.3 \pm 8.3	264
Open areas	26.5 \pm 9.2	7.7 \pm 5.7	168	37.3 \pm 14.5	9.6 \pm 9.0	260

such mechanisms to thermoregulation is much more important than the choice of what microhabitat they use.

Thus, our data produced three important results concerning the thermal consequences of microhabitat selection. First, habitat preferences did not change seasonally despite seasonal variations in the thermal environment. This is in contrast with previous studies that have found temperature-related seasonal shifts in microhabitat use (Huey *et al.*, 1977; Christian *et al.*, 1983; Christian & Bedford, 1995). Second, the observed pattern of selection was not always consistent with differences among microhabitats in thermal suitability. In both study seasons lizards used holm oak shrubs more often than expected on the basis of their availability, despite the fact that in May holm oaks had lower T_e s and larger d_e s than rockrose shrubs or open areas. Third, the importance of microhabitat selection as a thermoregulatory mechanism is probably less than previously thought, at least in temperate forests. We can therefore conclude that other aspects of the ecology of lizards should play a larger role than thermoregulation in determining the observed pattern of microhabitat selection.

Social behaviour might be one of these factors. However, social interactions in this species (territorial chases, courtship, mate guarding, copulations, etc.) take place only in spring. Thus, the observed constancy of microhabitat preferences cannot be attributed to the influence of social interactions. Prey availability might also influence microhabitat selection, because there may be more arthropods in the leaf litter beneath holm oaks, and this difference may be persistent between seasons. Another important factor might be predation risk. Mediterranean lizards in general, and *P. algirus* in particular, are important prey for a large number of predators, many of which can be found at the study area. Holm oak shrubs, with their prickled leaves and dense cover, could be selected for providing refuge against predators. Díaz

(1992) showed that the choice of compass directions around holm oaks allowed basking lizards to minimize the escape distance towards the nearest shrub, thus reducing predation risk. It has also been shown that the approach and escape distances of lizards from a deciduous oak forest were larger at the times of year when oaks were unleaved (Martín & López, 1995). Thus, predation risk could explain the preference of lizards for holm oaks and their avoidance of open areas (where dense herb cover might still offer some refuge) and especially of rockrose patches (with practically no cover at the ground level). Obviously, in summer the selection of holm oaks would also allow lizards to reduce the risk of overheating. We therefore suggest that microhabitat preferences, which did not change seasonally in the evergreen forest we studied, could be mainly related to antipredator behaviour rather than to thermal requirements.

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TABLE 2. Percentage of operative temperatures in each type of microhabitat that fell below, within, and above the lizards' selected thermal range. Sample sizes are shown in Table 1.

Microhabitat	May			July		
	Below T_{sel}	Within T_{sel}	Above T_{sel}	Below T_{sel}	Within T_{sel}	Above T_{sel}
Holm oak shrubs	79.9	8.0	12.1	56.5	14.2	29.3
Rockrose shrubs	66.8	12.9	20.3	39.8	12.5	47.7
Open areas	74.4	7.7	17.9	46.9	12.7	40.4

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THE HERPETOLOGICAL JOURNAL

Volume 15, Number 4 2005

CONTENTS

Papers presented at the BHS Scientific Meeting, 'Ecology and Conservation of British Snakes', 29 November 2003

Close encounters of the worst kind: patterns of injury in a population of grass snakes (*Natrix natrix*) P. T. GREGORY & L. A. ISAAC 213-219

Geographic variation in diet composition of the grass snake (*Natrix natrix*) along the mainland and an island of Italy: the effects of habitat type and interference with potential competitors L. LUISELLI, E. FILIPPI & M. CAPULA 220-230

Full papers

Taxonomic chaos in Asian ranid frogs: an initial taxonomic resolution L. CHEN, R. W. MURPHY, A. LATHROP, A. NGO, N. L. ORLOV, C. T. HO & I. L. M. SOMORJAI 231-243

The effects of shelter availability and substrate quality on behaviour and post-metamorphic growth in three species of anurans: implications for captive breeding P. T. WALSH & J. R. DOWNIE 245-255

Diverse types of advertisement calls in the frogs *Eupsophus calcaratus* and *E. roseus* (Leptodactylidae): a quantitative comparison R. MÁRQUEZ, M. PENNA, P. MARQUES & J. P. S. DO AMARAL 257-263

Egg mortality and early embryo hatching caused by fungal infection of Iberian rock lizard (*Lacerta monticola*) clutches P. L. MOREIRA & M. BARATA 265-272

The reproductive biology of *Salamandrina terdigitata* (Caudata, Salamandridae) F. DELLA ROCCA, L. VIGNOLI & M. A. BOLOGNA 273-278

Trophic eggs in the foam nests of *Leptodactylus labyrinthicus* (Anura, Leptodactylidae): an experimental approach C. P. A. PRADO, L. F. TOLEDO, J. ZINA & C. F. B. HADDAD 279-284

The role of relief in local abundance patterns of the spur-thighed tortoise *Testudo graeca graeca* in south-east Spain J. D. ANADÓN, A. GIMÉNEZ, I. PÉREZ, M. MARTÍNEZ & M. A. ESTEVE 285-290

Short Notes

An examination of *Pipa parva* (Anura: Pipidae) from native and invasive populations in Venezuela G. J. MEASEY & R. ROYERO 291-294

Seasonal changes in the thermal environment do not affect microhabitat selection by *Psammodromus algirus* lizards J. A. DÍAZ, S. CABEZAS-DÍAZ & A. SALVADOR 295-298