Collapse of the amphibian community of the Paul do Boquilobo Natural Reserve (central Portugal) after the arrival of the exotic American crayfish *Procambarus clarkii*

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Amphibian populations have suffered declines throughout the world due to factors such as habitat destruction, climate change, chemical pollution or the introduction of exotic species. The correct assessment of the declines, as well as of their causes, is dependent on sound monitoring protocols in selected areas, but medium and long-term studies including data before the declines are extremely rare. The red swamp crayfish, *Procambarus clarkii*, recently introduced to the Iberian Peninsula, is an efficient predator of amphibian embryos and larvae and recent reports have shown that its presence in a water body is a negative predictor of the presence of amphibian breeding populations. Here we compare data from a survey of the amphibian community of the Paul do Boquilobo Nature Reserve, central Portugal, made in 2001, with data gathered in 1992/93, just after *P. clarkii* arrived in the reserve. The first survey confirmed the presence of 13 species of amphibians in the reserve, four of them very abundant. In 2001, only six amphibian species were recorded, all in extremely small numbers. In only eight years, most of the previously abundant species strongly declined (e.g. *Pleurodeles waltl*, *Triturus marmoratus* and *Rana perezi*) or went locally extinct (e.g. *Hyla arborea* and *Pelodytes punctatus*). However, several species that disappeared from the main water body of the reserve continued to reproduce in nearby ponds without crayfish. Crayfish introduction seems the most probable cause for these declines. Therefore, the survival of several Iberian Peninsula amphibian populations may be dependent upon the correct management of the habitats not easily reached by the crayfish.

Key words: amphibian decline, long-term monitoring, invasive species, wetland

INTRODUCTION

The recent occurrence of declines among amphibian populations throughout the world is an unquestioned phenomenon (Blaustein et al., 1994; Baillie et al., 2004). An array of possible causes has been proposed for declines recorded in different species or world regions, from habitat destruction to climate change, chemical pollution or the introduction of exotic species (Blaustein et al., 1994; Beebee, 1996; Davidson et al., 2002). There have also been recent improvements in our understanding of the interactions between some of these possible causes (Blaustein & Kiesecker, 2002; Kats & Ferrer, 2003; Pounds et al., 2006; Rohr et al., 2008). The correct assessment of the declines, as well as of their causes, is dependent on sound monitoring protocols in selected areas, but medium and long-term studies are still relatively scarce (Young et al., 2001; Blaustein & Kiesecker, 2002). In particular, studies including data from before the declines are extremely rare (Beebee, 1996; Parker et al., 1999).

One of the identified causes of amphibian declines and extinctions is the introduction of exotic predators and/or competitors (e.g. Hecnar & M'Closkey, 1997; Rodríguez et al., 2005). Some of the clearest examples of extinctions driven by exotic predators are found after the introduction of exotic fish in previously fishless mountain lakes or streams (e.g. Knapp & Matthews, 2000; Fox et al., 2005). Exotic freshwater crayfish may have also been involved in declines, range restrictions and local extinctions of amphibians, mainly in lowlands in temperate climates (Axelsson et al., 1997; Nystrom et al., 2002; Witte et al., 2008).

The red swamp crayfish, *Procambarus clarkii*, is one of those species, and has been recently shown to be an efficient predator of amphibian egg masses, as well as of their larval stages in western Europe (Gherardi et al., 2001; Renai & Gherardi, 2004; Cruz & Rebelo, 2005; Cruz et al., 2006a). This species is native to the south-central U.S.A. and was first introduced in Europe at Doñana, southwest Spain, in 1973 (Habsburgo-Lorena, 1983). Its arrival in Portugal occurred in 1979 (Ramos & Pereira, 1981), probably through the Spanish tributaries of the Guadiana river, from where it quickly expanded across the southern regions of the country, locally reaching very high densities (Aquiloni et al., 2005). This was an area that had never maintained crayfish populations, thus the arrival of this functionally different predator and detritivore changed its trophic webs (Correia, 2001; Geiger et al., 2005; Rodríguez et al., 2005). Native communities were subjected to a pressure never experienced before; among amphibians, species that usually breed in temporary habitats (either ponds or streams) seem to be more vulnerable to this cray-

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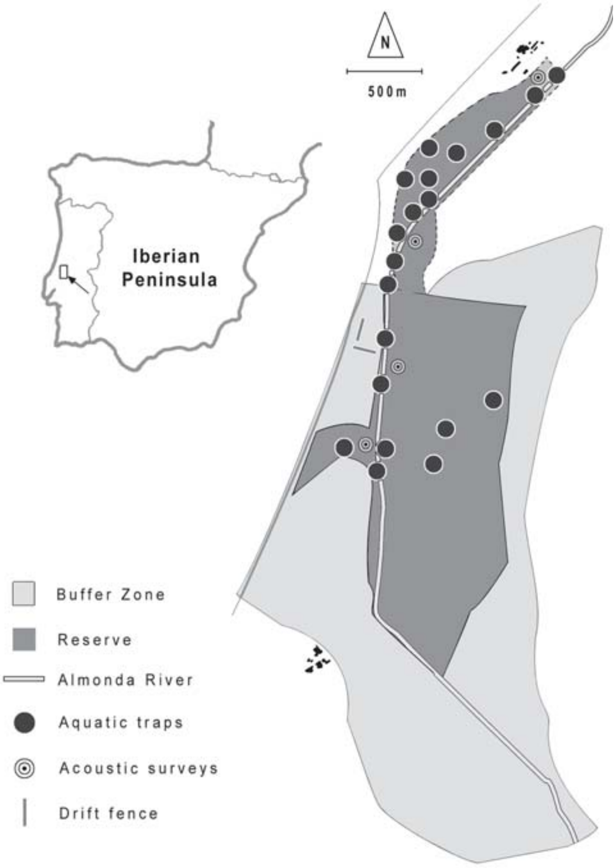

Fig. 1. Map of the Reserva Natural do Paul do Boquilobo (core area and buffer zone) with its location in the Iberian Peninsula. The headquarters are the black structures north of the reserve. The locations of the sampling sites are shown.

fish, and their use of a particular water body to reproduce is negatively related with crayfish presence (Cruz et al., 2006b).

The Paul do Boquilobo Nature Reserve (RNPB), central Portugal, covers the largest remaining marsh of the Tagus alluvial floodplain. This nature reserve was created in 1980 and was included in UNESCO's net of Biosphere reserves in 1981, its main natural interest being its egret and spoonbill breeding colonies. Since its creation, its vegetation and water levels have been managed to allow for the success of the bird colonies. As for its amphibian community, only isolated reports were available until the first thorough characterization was undertaken in 1992/93. This study confirmed the presence of 13 species of amphibians in the reserve (about three-quarters of the species present in Portugal), some of them very abundant, such as the warty and marbled newts, *Pleurodeles waltl* and *Triturus marmoratus*, and the common European treefrog, *Hyla arborea* (Segurado, 1994). This study laid the basis for an amphibian monitoring programme, and just happened to occur about two years after the arrival of *P. clarkii* to the RNPB. Since then, *P. clarkii* has maintained very high densities in the RNPB, reaching such extreme values as 15.6 individuals per m^2 (about 143 g of crayfish biomass per m^2; Cruz, 2001). Simultaneously, reports by the reserve wardens consistently pointed to a drastic reduction in the number of amphibians seen or heard in the main water body of the reserve.

In this work we present the results of a second sampling session conducted during 2001 (about 10 years after the arrival of *P. clarkii*), in which we repeated the sampling scheme of Segurado (1994), and assessed the status of the different amphibian species of the RNPB. We also assessed amphibian reproduction in nearby areas not yet colonized by the crayfish, and discuss the possible role of *P. clarkii* in the collapse of the amphibian community of this nature reserve.

METHODS

Study area

The Paul do Boquilobo Nature Reserve (RNPB) has an area of 554 ha and is located in central Portugal (39º23'N; 8º32'W), along the left margin of the Almonda river, a tributary of the Tagus (Fig. 1). The climate is classified as Mediterranean sub-humid, with mild rainy winters and dry, hot summers, with a variable summer drought. Climate data (average monthly temperatures and total monthly precipitation) for both sampling sessions were obtained from the closest meteorological station – Chouto, about 5 km SE of the RNPB) (Instituto de Meteorologia, Portugal). A large part of the reserve is flooded during the autumn and winter months; during the spring, the water is drained and some of the exposed terrain is used for agriculture. The drainage channels, as well as the banks of the Almonda river are covered with an exuberant riverine gallery forest, mainly of willow (*Salix* sp.), poplar (*Populus nigra*) and ash (*Fraxinus angustifolia*). The reserve is surrounded by flat agricultural fields to the south and east; to the north and west the terrain is more irregular and the small hills are covered with cork-oak (*Quercus suber*) woodland or olive-tree (*Olea europaea*) orchards. Scattered along this higher terrain there are temporary ponds and small streams that may become connected with the main water body during large floods.

Sampling

The sampling scheme of Segurado (1994) was repeated as closely as possible in 2001. However, due to time constraints, the 2001 sampling effort was in several occasions smaller than that of 1992/93.

Terrestrial habitats. 1992/93: Two sets of two perpendicular 20-m long drift-fences with 8 pitfall-traps each, checked at least three days per week from July 1992 to April 1993, in a total of 211 trapping days.

2001: One set of two perpendicular 20-m drift-fences with 8 pitfall-traps located in the same place as one of the previous sets (the one that registered the highest capture rates in 1992/93). This was checked 3 to 5 days per week in April, May, October, November and December 2001, in a total of 82 trapping days.

Acoustic surveys of calling anurans. 1992/93: At four sites spaced by at least 500 m (Fig.1), all calling anurans

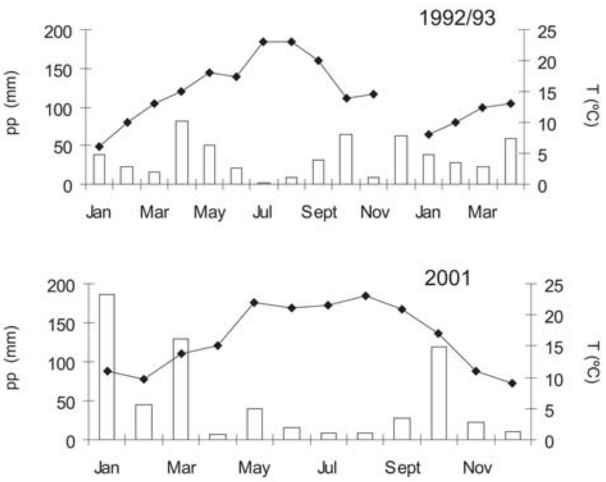

Fig. 2. Total monthly rainfall (pp, in mm) and average monthly temperatures (T, in °C) in each of the two sampling periods. Data obtained from the Chouto meteorological station (approximately 5 km SE of the RNPB), Instituto de Meteorologia, Portugal.

were recorded during a 5-minute period. Records were made twice per month from January 1992 to April 1993 at dusk, on a total of 26 nights. Calls were recorded according to species and in one of four abundance classes: 1 – none, 2 – a few (1–5), 3 – several (5–10), 4 – chorus (>10).

2001: The sampling scheme described above was repeated from March to December, on a total of 20 nights.

Aquatic habitats. 1992/93: 1–3 1.5 litre funnel traps were placed at each of 64 sampling points (the number of sampling points varied in each month, according to the water level), at least one day per month, from January 1992 to April 1993, on a total of 36 trapping days.

2001: 1–3 1.5 litre funnel traps were placed at each of 20 sampling points, 2–5 days per week, and checked daily from March to May and from October to December 2001, on a total of 26 trapping days. All of the sampling points from the 1992 study were repeated.

Pond surveys. These were performed systematically only in 2001. From January to December, we made monthly visits to 20 ponds within the RNPB and on the surrounding terrain (flat and hilly land), which were sampled with five three-metre sweeps of a dip-net (30 cm diameter; 2 mm green mesh). We identified all larval and adult amphibians.

Visual encounter surveys. These consisted of recording all the species found during the walks necessary for the placement and checking of the drift-fence and water traps. Sampling effort was not systematic, but contributed to the records of rare species.

Statistical analyses

In some cases, the sampling effort during 2001 was smaller than that of Segurado (1994). Therefore, we used a procedure for testing whether the 2001 results could be considered a random sample of the 1992/93 results. We produced 10,000 random samples and assessed the proportion of resamples containing the same or a larger number of selected taxa than the 2001 data. This procedure was applied to the results from the drift-fence traps (total anurans, total urodeles and all captures) and to the results from the aquatic traps (all amphibians). Data analyses were performed using S-PLUS 2000 (Statistical Sciences, 1999). In 1992/93, only the presence of *P. clarkii* in the funnel traps was recorded, and not the number of individuals. Therefore, the estimates of crayfish abundances used refer to the proportion of traps with crayfish. These were not normally distributed and were compared with Mann–Whitney U tests, with the program STATISTICA (version 6.0 for Windows).

RESULTS

Climate

The average monthly temperatures and total monthly precipitation in the RNPB during the sampling years are shown in Figure 2. Both 1992 and 1993 were drought years, with very low winter precipitation, while 2001 was a moderately dry year (average annual precipitation in

Table 1. Status of each amphibian species in the two sampling sessions. Methods used: a) drift-fence; b) acoustic surveys; c) aquatic traps; d) visual encounter surveys.

Species	1992/93	Based on	2001	Based on
Pleurodeles waltl	Very abundant	a, c, d	Rare; absent from the main wetland	d
Salamandra salamandra	Rare	d	Not found	
Triturus boscai	Rare	d	Not found	
Triturus marmoratus	Very abundant	a, c, d	Rare	a
Alytes cisternasii	Rare	b, d	Not found	
Discoglossus galganoi	Rare	d	Rare	a, d
Pelobates cutripes	Common	b, d	Rare; absent from the main wetland	d
Pelodytes punctatus	Abundant	a, b, d	Not found	
Bufo bufo	Rare	d	Not found	
Bufo calamita	Rare	b, d	Rare	a, d
Hyla arborea	Very abundant	b, c, d	Not found	
Rana perezi	Very abundant	a, b, c, d	Rare	b, d
Rana iberica	Rare	d	Not found	

Table 2. Results of terrestrial and aquatic sampling: number of captures per drift-fence per day and number of captures per water trap per day in the two sampling sessions. ("–": not applicable).

	Drift-fences		Water traps	
	1992/3	2001	1992/3	2001
Pleurodeles waltl	1.325	0	0.702	0
Triturus boscai	0.038	0	0	0
Triturus marmoratus	6.65	0	1.316	0
Discoglossus galganoi	0	0.024	0	0
Pelodytes punctatus	0.263	0	0.136	0
Bufo bufo	0.013	0	0	0
Bufo calamita	0	0.167	0	0
Hyla arborea	–	–	0.213	0
Rana perezi	1.513	0	0.307	0
Procambarus clarkii	0	0.155	–	–

RNPB approximately 800 mm; 1992 precipitation: 405 mm; 2001 precipitation: 617 mm). The 1992/93 drought was observed in both the autumnal and the spring rains.

Species richness

In 1992/93, 13 amphibian species were recorded at the RNPB. In 2001, only six species (four anurans, *Discoglossus galganoi*, *Pelobates cultripes*, *Bufo calamita* and *Rana perezi*, and two urodeles, *Pleurodeles waltl* and *Triturus marmoratus*) were recorded in the same area, in all cases in very small numbers. Table 1 depicts a simple abundance ranking of the 13 amphibian species of the RNPB in both study years, with an indication of the methods used to assess it.

Terrestrial sampling

Table 2 shows the captures at the drift-fences in 1992/93 and in 2001. In 2001 there were very few captures at the drift-fence: only one *T. marmoratus* individual and no *P. waltl*, *Pelodytes punctatus* or *R. perezi* (the more abundant species in 1993). For the other species, the capture rates were similarly low in both years. None of the 10,000 sub-samples of the 1992/93 results produced such low numbers, whether considering anurans, urodeles or the sum of individuals from both groups. During 1992/93, there were no captures of *P. clarkii* in the drift-fences; in 2001 these traps caught about 0.15 crayfish/trap/day.

Aquatic sampling

Table 2 depicts the captures at the funnel traps in 1992/93 and in 2001. No amphibians (adult or larvae) were captured in 2001, and once again, none of the 10,000 sub-samples of the 1992/93 results produced such low numbers, whether considering anurans, urodeles or the sum of individuals from both groups. In the autumn/winter season, the proportion of traps that caught *P. clarkii* per day was higher in 1992/93 than in 2001 (Mann–Whitney U-test, $z=3.45$; $P<0.001$); there were no differences in those proportions in the spring/summer between the two periods (Mann–Whitney U-test, $z=1.13$; $P>0.1$).

Acoustic surveys

The number of days with records of anuran calls and the modal abundance class of calling individuals of each of four vocally conspicuous species of the RNPB in both years are shown in Table 3. *Pelodytes punctatus* is an autumnal breeder and its calls were frequent in the autumn of 1992, being heard on six out of 20 occasions. This species was not heard in 2001. *Pelobates cultripes* is also an autumnal breeder that was heard in only one place (although in relatively high numbers) in 1992/93, and was not heard in 2001. *Hyla arborea* and *Rana perezi* are spring breeders at the RNPB, and their calls were particularly frequent in the springs of 1992 and 1993 (April–June), when choirs of tens to hundreds of individuals were heard. *H. arborea* was not heard during 2001, while *R. perezi* was still recorded on eight out of 23 occasions (comparable with the proportions in 1992 and 1993). However, the modal abundance class in 1992 and 1993 was the highest (choirs of >10 individuals), while the modal abundance class of 2001 was class 2 (isolated individuals).

Pond surveys

Out of the 20 ponds surveyed, significant numbers and diversity of amphibian larvae were only found in four, located in the hilly region north of the headquarters of the RNPB, about 300 m away from the main water body and not sampled during the 1992/93 study. There we found high numbers of tadpoles of several anurans: *Bufo* sp., *P. cultripes*, *P. punctatus* and *D. galganoi*, as well as larvae of *P. waltl*. In these ponds there were no captures of *P. clarkii*. All the other ponds were closer to the main water body and had *P. clarkii* on at least one occasion; amphibians were never found. These were the ponds where the highest densities of *P. clarkii* were recorded – up to 15.6 individuals per square metre, mostly juveniles (Cruz, 2001).

Visual encounter surveys

In 1992/93, amphibians were seen or heard on virtually all the walks in the RNPB. Particularly abundant species (such as *H. arborea* or *R. perezi*) were conspicuous and seen or heard very frequently in high numbers. In 2001, only four species were recorded this way: eleven adults of the previously very abundant *P. waltl* were observed on the roads surrounding the RNPB (always isolated individuals); three groups (10–20 individuals) of newly-

Table 3. Results of the acoustic surveys. Abundance classes were defined as follows: 1 – none, 2 – a few (1–5), 3 – several (5–10), 4 – chorus (>10).

	Modal abundance class		% of days with records	
	1992/3	2001	1992/3	2001
Pelodytes punctatus	2	1	25–50	–
Pelobates cultripes	3	1	0–25	–
Hyla arborea	4	1	75–100	–
Rana perezi	4	2	50–75	0–25

metamorphosed *P. cultripes* were found on the same roads; a few isolated *R. perezi* and *B. calamita* individuals were found in the agricultural fields south of the reserve. All these records were relatively distant from the main water body. In 1992/93, five rare species were found only during walks in the RNPB. With one exception (*D. galganoi*), none of these species was found in 2001 (Table 1).

DISCUSSION

In less than eight years, from 1992/93 to 2001, there was a collapse of the amphibian community of the RNPB, with a drastic reduction in the populations of *Pleurodeles waltl*, *Triturus marmoratus* and *Rana perezi* and the probable local extinction of previously abundant species, such as *Hyla arborea* and *Pelodytes punctatus.*

The effective sizes of some amphibian populations may fluctuate widely from year to year (Blaustein et al., 1994; Alford & Richards, 1999). Some species may even be absent from a particular location for several consecutive years, reappearing with favourable weather conditions. However, these patterns are more frequently found in unfavourable habitats or climates, whereas species breeding in permanent ponds are usually more predictable, reproducing typically every year (Pounds & Crump, 1994). We think that the drastic declines in the RNPB cannot be the result of stochastic population fluctuations, as 1) several species with different ecological requirements and life histories were affected; 2) for species with relatively short-lived individuals, like *H. arborea* (Barbadillo et al., 1999), eight years is a sufficiently long period to encompass one population turn-over; 3) 2001 was a wetter year than either 1992 or 1993, making it theoretically more favourable for amphibian sampling; and 4) this work was motivated precisely because we were alerted by local reserve wardens to the prolonged disappearance of the amphibians (especially of the night choruses).

The disappearance of several species from the main water body of the RNPB, together with evidence of continued amphibian reproduction in nearby ponds, suggests that the cause of the declines may be related to specific conditions in the main water body, and not to some alteration at the regional level. Furthermore, two of the species that were still recorded in 2001, *D. galganoi* and *B. calamita*, typically reproduce in ephemeral ponds, avoiding extensive, permanent waters (Díaz-Paniagua, 1990), and thus were probably reproducing in the ephemeral ponds not connected to the RNPB's main water body.

Among the multiple factors that may influence amphibian populations, only two have significantly changed between 1992 and 2001 in the main water body of the RNPB: water quality and the establishment of *P. clarkii*, although the possibility remains that some other unaccounted factor may be involved in the declines registered. As far as water quality is concerned, the reserve has received the effluents of some nearby towns and agro-industrial plants since the 1970s and was commonly polluted by free ammonia, phosphates and faecal coliforms (Sousa, 2001). However, the waters were kept sufficiently clean for fishing to be allowed, and local fishermen sell their products for local human consumption. Water quality has been monitored by the reserve authorities and there was an improvement of its quality from 1999 to 2001 (Sousa, 2001).

When Segurado began the first assessment of the RNPB's herpetofauna, *P. clarkii* had just arrived in the area. This is a colonizing species, with a capacity to rapidly build up large populations (Cruz et al., 2004), due to its omnivorous diet, high fecundity and maternal protection of the newly-hatched offspring. In spite of its presence in the Iberian Peninsula for over 30 years now, only recently have there been reports of *P. clarkii* preying on native amphibians: this species was able to actively prey on free larval stages of all 13 species of the RNPB, the majority of them with no evolutionary history of contact with crayfishes. The consumption rate of amphibian egg masses by this species was very high in mesocosm experiments, even when alternative vegetable food items were offered (Cruz & Rebelo, 2005). In a one-day field experiment undertaken at Doñana Natural Park (southwest Spain), over 90% of the egg masses of *B. calamita* were consumed by *P. clarkii* in just one night (Cruz et al., 2006a). Finally, Segurado (1994) had already noticed that some adult *P. waltl* exhibited deep wounds in their tails or limbs; such occurrences are now common in places where urodeles and *P. clarkii* coexist (Cruz, pers. obs.).

The disappearance of amphibians from crayfish-invaded waters has also only recently been reported (Rodríguez et al., 2005; Cruz et al., 2006a). Another recent work showed that in southwestern Portugal there is a negative relationship between the invasion by *P. clarkii* and the probability of use of a particular water body by a set of native amphibians, particularly urodeles, *P. cultripes*, *B. bufo* and possibly *Pelodytes ibericus* (Cruz et al., 2006b). Whether this negative relationship is due to avoidance by adult amphibians of habitats colonized by *P. clarkii*, or to the rapid consumption of egg masses or free-swimming larvae by the crayfish, was not clear, but both factors may contribute to the absence of a particular amphibian species in ponds with crayfish. The similarity among the lists of most affected species between that and the present study may indicate that the same causative actor is involved, namely the arrival of this exotic predator.

The catastrophic amphibian declines in the RNPB may result from a set of characteristics that confer on this area optimal conditions for crayfish maintenance and reproduction: this is a relatively shallow wetland that maintains free water all year round, it is located in a benign climate for *P. clarkii* (water never freezes and is frequently above 10 ºC) and has high availability of decaying vegetation. The clay soils permit easy burrowing to a wet layer during the short adverse period (late summer). Furthermore, the main wetland is surrounded by a series of vegetated smaller ponds that are created during the floods. These are devoid of predatory fish and maintain high densities of *P. clarkii* (especially juveniles). As in other areas, this kind of pond may function as nurseries for the crayfish and allow the repeated colonization of the main water body by adult cohorts (Correia, 1995; Cruz et al., 2004).

Crayfish species have been transferred around the globe producing major impacts in many of the freshwater ecosystems where they have been introduced (e.g. Hobbs et al., 1989; Gutiérrez-Yurrita et al., 1998; Lodge et al., 2000a; Smart et al., 2002), and are now recognized as responsible for some amphibian declines (Kats & Ferrer, 2003). Consequently, several countries have created appropriate legislation to prevent future introductions (Lodge et al., 2000b; Huner, 2002). However, these regulations have proved difficult to enforce and have not prevented the introduction of *P. clarkii* throughout Europe (Huner, 2002). In Portugal, the introduction and detention of invasive alien species has had specific legislation since 1999 (Decreto de Lei n.º 565/99 from 21/12; Declaração Rectificação n.º 4 – E/2000 from 31/01). This national legislation establishes a generic prohibition to intentional introductions and has annexed a list of invasive alien species that includes *P. clarkii*. Unfortunately, *P.clarkii* had already spread throughout most of the country before this law was created. To further complicate things, an effective means to completely eradicate a *P. clarkii* population has not yet been described (Barbaresi & Gherardi, 2000; Kerby et al., 2005), as the use of xenobiotic chemicals and non-ionic surfactants has proved to be ineffective (Anastácio & Marques, 1995; Anastácio et al., 2000; Huner, 2002).

During the last decade, there has been a complete alteration in the RNPB food web. *P. clarkii* is now known to be the main food item of the egret, spoonbill, stork and tern colonies; its remains appear in the scats of all the carnivores of the RNPB, and even in those of wild boars (Sousa, unpubl. data). Moreover, during a three-year period (2000–2002), about 10 local fishermen were allowed to capture crayfish in the reserve and removed an estimated two tons of crayfish per fisherman per year (Sousa, unpubl. data). Still, crayfish abundance remained high, and their eradication in the main RNPB water body seems very unlikely.

All the amphibians of the Iberian Peninsula are dependent on aquatic habitats to complete their larval phase, which means that crayfish introductions may have a highly significant impact on these species, maybe even causing their local extinction in specific areas, such as permanent wetlands like the RNPB. In the future, local authorities should enforce the prohibition on introducing *P. clarkii*; in areas already colonized by this species, the survival of amphibians may be dependent upon the correct management of the habitats not easily reached by the crayfish, such as temporary ponds and high stream riffles (Cruz & Rebelo, 2007).

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