Rainfall stochasticity controls the distribution of invasive crayfish and its impact on amphibian guilds in Mediterranean temporary waters

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Abstract Invasive crayfish have severely impacted invaded aquatic ecosystems worldwide. We studied temporal and spatial variation in the range expansion of the red swamp crayfish at one of the first European localities to which it was introduced: Doñana National Park (SW Spain). In contrast to the rapid range expansion witnessed in other areas, this invasive crayfish has not spread across the entire park. Instead, its distribution has expanded during wet periods, but contracted during drought periods. The red swamp crayfish has caused steep amphibian declines in other invaded areas. However, after approximately 35 years of crayfish presence in Doñana National Park, we have yet to detect a reduction in the number or occurrence of amphibian species. Amphibians may thus be protected by the large abundance of temporary ponds in the area, which provides them with an effective refuge network. We show that natural fluctuations in annual rainfall and in the number of ponds filled can temporarily eliminate invasive crayfish from particular areas. This fact should be taken into account when attempting to reduce the impact of crayfish on aquatic communities, intensifying crayfish removal during particularly dry years, when it is most effective.

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Introduction

Invasive species may severely impact invaded areas by altering the structure of native communities (Simberloff, 2005). Freshwater ecosystems worldwide have experienced a high number of biological invasions, with invaders stemming from diverse taxa: introduced fishes, invertebrates, plants, or even microorganisms have all had strong impacts on native communities and, at times, caused the extinction of native species (see reviews by Moyle & Light, 1996; Ricciardi & MacIsaac, 2011). Amphibians are the most threatened group among vertebrates (Hoffmann et al., 2010), and their aquatic larvae and eggs are the life stages most vulnerable to predators. One of the main causes for such global amphibian decline is the introduction of alien species (Kieseker, 2003; Blaustein et al., 2011). The most damaging alien taxa to amphibian populations are fishes, crayfish, other amphibians, and snakes (Wells, 2007), and they are mainly found in permanent waters. Therefore, amphibian species that typically breed in permanent aquatic habitats are considered to face greater risks from alien predators than those breeding in temporary ponds (Kats & Ferrer, 2003). The impact of predation is magnified because amphibians often fail to recognise alien predator cues and thus their antipredator defences are not triggered (Kats & Ferrer, 2003; Gomez-Mestre & Diaz-Paniagua, 2011). Temporary ponds are aquatic habitats from which top predators are usually absent; as a result, they are secure reproductive sites for many amphibian species (Semtlisch, 2003). However, temporary ponds are also highly fluctuating habitats that may experience widely varying hydroperiods depending on the amount of rainfall they receive (Brooks, 2004). These ponds may also occasionally increase in size during inordinately heavy rains, which can increase their connectivity with temporary or permanent habitats, and thus enable the inflow of top predators. Spates of exceptional floods have been reported to favour the expansion of alien crayfish in invaded areas in Portugal (Bernardo, 2011).

The impact of the red swamp crayfish has been documented in detail, and the species has been found to affect organisms at different levels of the food web in aquatic ecosystems (Geiger et al., 2005; Gherardi, 2007) Its introduction into aquatic habitats has produced drastic changes in invaded ecosystems, reducing the complexity and structure of their food webs (Geiger et al., 2005). In addition, *P. clarkii* is a significant predator of amphibian eggs and larvae (Cruz & Rebelo, 2005; Portheault et al., 2007). After its introduction, severe reductions in amphibian species were documented in certain areas of Portugal (Cruz et al., 2008) and Spain (Rodríguez et al., 2005). Nonetheless in Doñana National Park, crayfish have been reported to have had a less drastic impact on amphibians, despite this area having been invaded earlier than the other Iberian areas studied (Díaz-Paniagua et al., 2006).

In this study we analyse the effect of an invasive crayfish species on an amphibian community in a well-protected area. We analysed the temporal and spatial variation in the distribution of crayfish, starting at the time of its initial introduction to Doñana National Park, one of its first introductions in Europe. Because most of the aquatic habitats in this area are temporary ponds and their filling is associated with rainfall, we first predicted that crayfish distribution in the park could also vary in relation to annual variations in rainfall, especially in years of severe drought. Using data from field surveys of aquatic systems carried out from 1978 to 2010, we first evaluated the temporal variation in the distribution of this species in relation to the annual rainfall recorded in the park, a variable that directly reflects differences in the annual inundation of the park's ponds. Similarly, changes in amphibian occurrences would be expected, and as in other areas, they could potentially be related to variations in crayfish occurrence. Thus, our second aim was to evaluate the species' co-occurrence with amphibian guilds in order to detect changes in amphibian occurrence between periods previous and posterior to a very dry year, since dry years reduce the availability of aquatic habitats, which in turn could affect the stabilisation of crayfish populations. We also predicted that the crayfish invasion would have caused a decrease in amphibian species richness and occurrence in the park and our third aim was to compare data on amphibian occurrences over the course of our 30-year sampling record.

Methods

Study area

Doñana is located in southwestern Spain, between the Atlantic Ocean and the mouth of the Guadalquivir River. The park extends across 54,252 ha and is divided in two well-differentiated geomorphological units: a marshy area with a clay substrate and an aeolic sandy system composed of moving and stabilised dunes (Siljeström et al., 1994). The northern sandy area is characterised by stable dunes covered by Mediterranean heath and contains a high density of temporary ponds that serve as the primary breeding sites for amphibians (Díaz-Paniagua et al., 2006). The marshland and the temporary ponds become flooded in autumn or winter, and dry out during the summer. Therefore, we report annual periods in terms of the hydrological cycle, from September of a given year to August of the next year.

Within the park there are only two large permanently flooded ponds, which are located in the contact area between the stable dunes and the moving dunes (Peridune area; Fig. 1A). These ponds dried up after multiple years of severe drought (between

1991 and 1995). Alternatively, these ponds can become temporarily connected with the marsh in years of abundant rainfall (as was the case in 1987-1988, 1989-1990, 1995-1996, 1996-1997, 1997-1998, 2000-2001, 2003-2004, and 2009-2010, when annual rainfall exceeded 700 mm). During episodes of heavy rainfall, small intermittent streams may connect ponds and flow towards the marsh (Fahd et al., 2007; Díaz-Paniagua et al., 2010; Gómez-Rodríguez et al., 2011).

During summer droughts, water only persists in a few channels and deeper basins within the marsh, at the mouth of the streams opening to the marsh, and in small artificially deepened ponds ("zacallones"). Zacallones are often excavated within the basin of natural ponds and ensure water availability for cattle and wildlife during the summer; they are scattered throughout the sandy area of the park.

The contact area between the stable dunes and the marsh (marsh edge) is a long and narrow zone characterised by meadows and ferns in which small temporary streams and a large number of small and shallow ponds are annually filled. In the southern sandy area, beyond the peridune area, there are moving dunes interspersed with valleys and stabilised dunes covered by an extensive pine forest. Temporary ponds are not as abundant in this area as in the north, and the main aquatic habitats are isolated zacallones. In the centre of the park, a protected area of 8,000 ha, the Doñana Biological Reserve, has been especially set aside for scientific research; here, aquatic sampling has been more intensive than in the rest of the park (Fig. 1 A). A more detailed description of the system of temporary ponds in Doñana is given in Díaz-Paniagua et al. (2010).

Crayfish introduction

Invasive crayfish have been introduced into many countries for commercial purposes, where they have subsequently invaded aquatic habitats (Gherardi, 2007; Peay, 2009).

The red swamp crayfish, *Procambarus clarkii* (Girard 1852), was first introduced to Europe in 1973, to the Extremadura region of Spain and then again in 1974, to the marshes of the Guadalquivir River (Habsburgo-Lorena, 1978). In this marshy area, crayfish were reared in aquaculture facilities from which they escaped and spread into natural environments, including the Doñana National Park (Habsburgo-Lorena, 1978). Over the last 30 years, the red swamp crayfish has been introduced in a total of eleven European countries (Holdich et al., 2010).

Crayfish and amphibian data

In this study, we have combined data from different monitoring programs designed to survey various aquatic organisms (amphibians, macroinvertebrates, turtles and crayfish). These programs were carried out from 1978 to 2010 in different areas of Doñana National Park, and they all recorded the presence of crayfish. We used crayfish data obtained in three monitoring programs: The first program involved surveys focused on amphibians. From 1978 to 1988, 2002 to 2004, and 2005 to 2007, monthly sampling of amphibian larvae was carried out in 8 to 22 temporary ponds within the Doñana Biological Reserve. Additionally, from 2002 to 2004 and 2005-2007, we annually sampled 189 to 332 ponds throughout the whole park to collect data on amphibian larvae. Sampling was conducted via dipnetting: a stretch of approximately 1.5 m of water was swept at different points within each pond and the number of amphibian larvae present was recorded (see details in Gómez-Rodriguez et al., 2010a). In these annual surveys, we also recorded the presence of aquatic macroinvertebrates (Florencio et al., 2013). The second program involved surveys focused on aquatic turtles. These surveys took place from 1983 to1988 in the Doñana Biological Reserve; from 1992 to

2000, sampling also took place along the border of the marsh as well as in ponds and streams at the northern edge of the park. Sampling was conducted using baited fyke traps in 9 to 68 ponds. These traps were active for 24-hour periods. The third program involved surveys focused on crayfish. A standardised trapping protocol was used to monitor crayfish from 2004 to 2010 in 50 ponds in the sandy area, as well as at 22 locations within the marsh. These data come from crayfish and amphibian monitoring programs periodically carried out by the Natural Processes Monitoring Team of the Doñana Biological Station (CSIC) (www.rbd-ebd.csic.es/Seguimiento). Since these programs were, in some cases, not specifically designed to survey crayfish, there are important methodological differences among them in their sampling scheme approaches and techniques. Therefore, they do not provide standardised and comparable data on crayfish abundance. Hence, we only used crayfish presence data when assessing temporal variation in crayfish range in Doñana National Park. Sampling programs enacted before 1988 were only carried out in the central area of the park and do not provide precise information about crayfish range expansion during those years. However, they do provide the first records of crayfish presence in sandy areas and temporary ponds.

Data analyses

We determined the distribution of the red swamp crayfish in Doñana National Park by pooling all the presence data across the entire study period. However, in order to unambiguously assess variation in crayfish presence over time and minimise the likelihood of false absences (i.e. failure to detect the crayfish at a site where it was actually present), we distinguished data from sites that had been sampled in at least three different years (often several times per year). For these sites, the presence of the crayfish was categorised according to the number of years in which it was detected: always present (permanent), occasionally present (absent from at least one of the sampling years), or always absent.

Different aquatic sampling techniques, however, can yield different representations of aquatic communities (Florencio et al., 2011). Therefore, for the sake of consistency, we restricted our analyses of interannual variation in crayfish range to the two data sets for which sampling had been done using fyke nets: from 1990 to 2000 (aimed at aquatic turtles), and from 2003 to 2010 (aimed at crayfish and aquatic turtles). The number of sites sampled differed widely throughout the study period; we therefore corrected the presence data for the number of sites sampled each year (i.e. n sites with crayfish/ n sites sampled), and also expressed this presence as a percentage. We used generalised linear models to quantify the effect of rainfall on crayfish frequency of occurrence within the park. We modelled the presence/absence of crayfish as a function of the precipitation of the current year, the previous year, or both, fitting a binomial error distribution and a logit link function with R (Core Development Team R 2011). We assessed the goodness-of-fit of these models using the Akaike Information Criterion (AIC): $AIC = -2LnL+2k$, where k is the number of parameters in the model and LnL is the log likelihood (Burnham & Anderson, 2002). Differences in AIC values between models were considered negligible if they were less than 3, very strong if they were greater than 10, and moderately strong if they were between 4 and 7 (Burnham & Anderson, 2002).

Crayfish occurrence is expected to be reduced after drought periods, which could in turn increase the occurrence of amphibians. We tested whether the occurrence of amphibians varied after a period of drought, throughout different areas of the park.

To this end we used data from two periods of intensive amphibian surveys in consecutive years: 2002-2004 and 2005-2007. These two sampling periods were separated by a very dry annual period 2004-2005, during which neither temporary ponds nor marshlands were flooded in Doñana. Of the 372 sites sampled from 2002 to 2007, 157 were sampled during both periods. The occurrence of amphibian species in the ponds of Doñana presents a characteristic dynamism, with large differences even between consecutive years. Thus, single-year surveys are insufficient to determine the species assemblage associated with any given pond (Gómez-Rodriguez et al., 2010a). Therefore, in order to increase the accuracy of amphibian data for particular time periods, we grouped amphibian presence data in 2-year blocks. We counted the number of ponds in which each amphibian species was present to estimate the amphibian percentage of occurrence (100*number of sites at which target species was present divided by the total number of sites) in the pre-drought (2002-2004) and post-drought (2005-2007) periods. Differences in amphibian percentages of occurrence between the periods were compared using Wilcoxon signed rank tests for paired data. With this non-parametric test, we compared the number of ponds in which all amphibian species were detected before and after the dry year for each zone of the park.

In order to evaluate changes in amphibian occurrence that could have been influenced by crayfish introduction, we used our earliest sampling data. These were obtained in 1978-1979 and 1979-80, for eight temporary ponds in which we monitored amphibian larvae. Five of these ponds were also monitored during three later periods of time (1983-1984 and 1984-1985; 2002-2003 and 2003-2004; 2005-2006 and 2006-2007), enabling us to assess the variation in amphibian presence at these sites after crayfish invasion. In order to have a similar number of ponds across periods, we considered for the three later periods, data from three additional temporary ponds whose characteristics and locations were similar to those three ponds sampled in 1978-80 which had not been monitored in later years. The number of ponds in which each amphibian species was present was compared across successive periods using Wilcoxon signed ranks tests for paired data; we adjusted the significance level of the tests using Bonferroni corrections, where the alpha level was set to $p<0.0125$.

Results

Spatial variation in crayfish occurrence

The cumulative distribution of *P. clarkii*, including data from 1983 to 2010, revealed that this invasive species is found mainly in the northern half of Doñana National Park (Fig. 1B). The crayfish has expanded its range and is now found throughout the whole marshland and the northern sandy area of the park. Permanent crayfish presence was detected along the marsh edge and in locations where water persisted all year long, e.g. streams and excavated ponds (zacallones) in the peridune and northern sandy areas (Fig 1B). Crayfish were also present in 59.5% of the sites sampled throughout the innermost marsh areas, which are usually dry during summer. In the northern sandy area of the park, crayfish were present in 28.1% of the ponds, mostly excavated permanent ponds (56%) or long-lasting temporary ponds (40%) in which crayfish only appeared occasionally. The crayfish's percentage of occurrence (88.9%) was also high in both permanent and temporary ponds in the peridune area. In contrast, crayfish were never captured in the southernmost sandy area of the park (Fig. 1B, Table 1).

Annual variation in crayfish range (1978-2010)

From 1978 to 1982, only water bodies in the central area of the park were sampled. These samples revealed that no crayfish were present in the sandy areas or at the marsh's edge. *Procambarus clarkii* was first detected in 1983-1984, in two semi-permanent water bodies at the marsh's edge. In subsequent years, crayfish were also found in six additional ponds bordering the marsh and were first observed to occupy ponds within the sandy area in 1988, a very rainy year (Fig. 1A). Crayfish distribution within the park showed remarkable interannual variation from 1992 to 2010 and was dependent on the amount of rainfall recorded (Fig. 2). Crayfish presence at any given time was strongly affected by the same year's precipitation (Null model: AIC = 152.87; model fitting same year's rainfall: AIC = 133.98, 22.7% deviance explained) and moderately affected by the precipitation of the previous year (AIC = 148.32, 7.1 % deviance explained). The best fitting model, however, included both the same year's rainfall and that of the preceding year (AIC = 122.18, 37.6% deviance explained).

In figure 3, we observe in detail, how the presence of *P. clarkii* declined gradually from 1992 to 1995, a period that included years of severe drought. In contrast, a new expansive pulse of crayfish was detected immediately after 1995-1996, which was a very rainy annual period (annual rainfall =1032 mm). Similarly, reductions in crayfish expansion were subsequently observed in very dry years (e.g. 1999 and 2005; Fig. 2), as crayfish became restricted to the few remaining zacallones.

Crayfish impact on amphibian guilds

We assessed the effect of drought, which would also contract crayfish range, on amphibian occurrence by comparing data separated by a very dry year. Fig. 4 shows the percentage of occurrence of each species of amphibians in the sampled ponds before and after a severe dry year. All ten species of amphibians were recorded in pre-drought (2002-2004) and post-drought (2005-2007) sampling periods. *Pelophylax perezi* (López Seoane, 1865), *Pelobates cultripes* (Cuvier, 1829), and *Pleurodeles waltl* Michahelles, 1830 were the amphibian species with the highest co-occurrence with *P. clarkii*. During the pre-drought period, crayfish co-occurred with most amphibian species in different areas of the park, except in the southern ponds. However, during the post-drought period, crayfish were also absent from peridune ponds (Fig. 4). We observed significant differences in amphibian occurrence between both periods across all areas (marshes: Wilcoxon test, Z=2.24, P=0.0251; marsh edge: Wilcoxon test, Z=2.803, P=0.0051; northern sandy area: Wilcoxon test, Z=2.803, P=0.005; peridune ponds: Wilcoxon test, Z=2.803, P=0.005, southern ponds: Wilcoxon test, Z=2.073, P=0.038), indicating that these differences were not uniquely attributable to the impact of crayfish. After the drought, the crayfish percentage of occurrence slightly decreased in the marshes (59.9% to 44% in pre- and post-drought respectively), whereas we observe in Fig. 4 that the percentage of occurrence of most amphibian species increased (Fig. 4a). In the remaining areas, despite differing in general occurrence between periods, most amphibians persisted and demonstrated high percentages of occurrence. One exception was *Pelodytes ibericus* Sánchez-Herráiz, Barbadillo, Machordom & Sanchíz, 2000, which is common in the marsh but not in temporary ponds. Only at the marsh's edge, where co-occurrence with crayfish increased after the drought, we observed a decrease in most of the species observed, except for *P. cultripes* and *P. perezi* (Fig. 4b).

The eight ponds monitored in subsequent periods from 1978 to 2007 revealed a large but non-significant degree of variation in amphibian percentages of occurrence during these years (Table 2, Wilcoxon rank test). The presence of *P. clarkii* in these ponds also varied substantially among years. Only marginal differences (after Bonferroni corrections) were detected between the first and the last sampling period (Table 2, Wilcoxon rank test after Bonferroni corrections, $P = 0.028$). A gradual reduction in *P. perezi* was observed in these ponds over time, whereas *P. cultripes* did not demonstrate large differences in occurrence. The highest occurrence of *Lissotriton boscai* (Lataste, 1879) and *D. galganoi* Capula, Nascetti, Lanza, Bullini & Crespo, 1985 were recorded in the years prior to crayfish introduction to the sandy areas of the park (Table 2). For the other species, we observed no trends associated with the presence of crayfish.

Discussion

Invasive crayfish do not occupy all aquatic habitats

More than 30 years after its introduction, *Procambarus clarkii* is widely distributed across Doñana National Park, but it has not invaded all of the park's aquatic habitats. In fact, it is mainly present in permanent or long-lasting water bodies, and it only reaches temporary ponds (the main breeding sites for amphibians) following years of high precipitation.

While crayfish are common in permanent waters and breed asynchronously in their original habitats in North America, the introduced populations in Doñana have modified their annual cycle in order to resist the summer drought. They dig deep burrows to reach wet soil, where they mate, spawn, and hatch. These burrows are also used as refuges during the summer as the crayfish wait until the next flooding event occurs (Bravo et al., 1994); hence the crayfish recolonise the wide expanse of the marsh every year. Burrow-building is thus key to the persistence of crayfish populations in Mediterranean wetlands. In Portugal, an adequate silt/coarse substrate was also described as being a required feature of crayfish habitats (Correia & Ferreira, 1995). The impermeable clay and silt substrate of the Doñana marshes may favour the construction of such refuges. In contrast, the sandy substrate of other areas of the park does not allow burrow construction, and there we observe that crayfish are constrained to permanent ponds, most of which are ponds that are excavated to water cattle and wildlife over the summer.

The dispersal capacity of crayfish increases during periods of high floods (Bernardo et al., 2011). In very rainy years, connectivity among aquatic habitats is increased in our study area, as water bodies become temporarily connected in the northern area of the park, thus favouring the expansion of the crayfish's distribution. Colonisation of temporary ponds adjacent to summer refuges is thus dependent upon the proximity of the water bodies and their connectivity. Díaz-Paniagua et al. (2010) estimated 483 ponds formed in the southern part of the park in a rainy year, in contrast to 2886 ponds in the northern sandy area. In contrast, the lower density of water bodies in the south may be the reason why crayfish have never reached this area, in which the connectivity necessary to dispersal is unlikely to occur.

Models of crayfish distribution in invaded areas of the Iberian Peninsula and Italy reveal the importance of permanent water bodies in population persistence, while the colonisation of temporary waters depends on water body distance from crayfish source populations (Cruz & Rebelo, 2007; Siesa et al., 2011). The high interpond distances in southern Doñana have safeguarded this area from crayfish invasion. As a result, it acts as an important sanctuary for particular plant (Díaz-Paniagua et al., 2010) and amphibian populations that have never co-occurred with this invasive crayfish (Gomez-Mestre & Díaz-Paniagua, 2011).

Drought periods reduce the range of the invasive crayfish

After its introduction in 1974 to the Doñana marshes, *P. clarkii*'s spread was favoured by its colonisation of adjacent rice fields, where water management practices allowed the species to have a reproductive cycle with up to 3 generations per year (Cano & Ocete, 2000). The species then expanded throughout the marsh, where it had to withstand the marked seasonality of the region's harsh, dry summers. Unpredictable and large fluctuations in pond area, water duration and physico-chemical variables are common features of temporary ponds (Gómez-Rodríguez et al., 2010b; Díaz-Paniagua et al., 2010). Although *P. clarkii* is able to survive summer conditions in the Doñana marshes by withdrawing to deep wet burrows, the occurrence of years of severe drought has reduced the number of sites with adequate habitat and greatly contracted the species' range. The complete desiccation of most aquatic habitats during drought periods explains the reduction in crayfish range in dry years. In addition, other factors may have contributed to the decreased size of the crayfish population during dry years (during which individuals are concentrated at a few sites), such as an increase in predation intensity and the impoverishment of food resources. Extremely dry years occurred in 1994-1995, 1998-1999 and 2004-2005. In 2004-2005 in particular, we detected a considerable general decline in crayfish; they even disappeared from particular areas, e.g. the peridune area with its large permanent ponds, where they had previously thrived. Therefore, although Doñana has been widely colonised by this invasive crayfish, the species does not find optimal habitats throughout the entire park, and its populations have experienced pulses of expansion and contraction in response to the rainfall fluctuations that are typical of the Mediterranean climate.

Impact of the crayfish on amphibians

Amphibian populations have been reported to decline following the introduction of the red swamp crayfish in areas of Portugal (Cruz et al., 2006) and Italy (Ficetola et al., 2011), despite these areas having been invaded later than Doñana (Rodríguez et al., 2005; Cruz et al., 2008; Ficetola et al., 2011). More than 30 years after the introduction of red swamp crayfish, however, we can still find well-preserved populations of amphibians in Doñana (Díaz-Paniagua et al., 2005, 2006). The abundance and heterogeneity of protected temporary ponds in the park result in a robust habitat network for amphibian reproduction that helps to preserve a diverse amphibian guild (Fortuna et al., 2006; Díaz Paniagua et al., 2006).

Although the red swamp crayfish preys upon the eggs and larvae of all the amphibian species present in Doñana (Cruz & Rebelo, 2005; Portheault, 2010), the crayfish is likely to pose different levels of risk to different amphibians depending on their breeding phenology and habitat preferences. Thus, species with short larval periods may successfully breed in ephemeral habitats of short hydroperiods, areas in which crayfish rarely occur (e.g. *Bufo calamita*; Portheault et al., 2007). In contrast, species that require long hydroperiods or permanent ponds have a much higher probability of encountering invasive crayfish (Gomez-Mestre & Díaz-Paniagua, 2011). In fact, the species requiring long hydroperiod ponds (*Pelobates cultripes*, *Pleurodeles waltl* and *Pelophylax perezi*, after Díaz Paniagua,1988) most frequently coexisted with crayfish. We have found no evidence for significant variation in amphibian occurrence over time, indicating that, apparently, no local amphibian extinctions have yet occurred due to the invasive crayfish. However, strong crayfish predation on amphibian eggs and larvae may have decreased recruitment in local populations. Further analyses of genetic diversity in amphibian populations with different degrees of historical exposure to the invasive crayfish would clarify whether the crayfish has already had an impact despite not having caused local extinctions yet.

Much of the observed population variation is associated with the common interannual dynamism previously described in this amphibian community (Gómez-Rodríguez et al., 2010a). Depending on the quantity and timing of annual rainfall, the same temporary ponds may offer different breeding opportunities for amphibians; they may be adequate for successful reproduction of different amphibians in different years, and the resultant alternation of species may, in the long term, favour amphibian diversity across the whole area (Gómez-Rodríguez et al., 2010a).

The episodic occurrence of severe drought events may explain why crayfish have had a less catastrophic impact on amphibian communities in Doñana than on communities in other invaded areas in Europe. The inability of *P. clarkii* to have a stable presence in most temporary ponds has reduced the damage that it could have inflicted upon amphibians. The amphibian guild in Doñana as a whole still maintains the temporal turnover of species (Gómez-Rodríguez et al., 2010a), although the fluctuating presence of the red swamp crayfish undoubtedly constitutes an important additional mortality risk. Crayfish occurrence increases in years of abundant rainfall, when the number of temporary ponds notably increases as well (Gómez-Rodriguez et al. 2010b), and therefore amphibians also have access to more breeding sites. At particular sites, however, the impact of the invasive crayfish may be high. When a given pond is colonised by crayfish, tadpole abundance is markedly reduced, especially in latebreeding species with long larval periods like *P. perezi* (Díaz-Paniagua & Gomez-Mestre, pers. obs.). Slowly developing tadpoles, like those of *P. cultripes*, are also at risk even though they attain large sizes; we have also observed high incidences of severely injured tadpoles.

Although crayfish invasion has not resulted in a clear reduction in amphibian richness or frequency of occurrence in Doñana National Park, it is worth noting that some species demonstrated their highest occurrences prior to the crayfish's arrival in the ponds of the sandy area of the park. For example, although the three species with the longest larval periods (*P. waltl*, *P. cultripes* and *P. perezi*) are still very abundant in this area (Díaz-Paniagua et al., 2006), they seem to be less present in permanent habitats or in the marshes. For *P. waltl* and *P. cultripes* in particular, explosive events in which thousands of individuals emerged and moved towards their breeding sites during autumn or winter days were common in Doñana 30 to 40 years ago (Valverde, 1967; Díaz-Paniagua *pers. obs*.); however, they have not been observed since 1990 (Díaz-Paniagua et al., 2005).

Implications for conservation

Eradication or control of this invasive crayfish species is presently considered unlikely in most of the invaded areas, despite strong recommendations in favour of limiting their expansion or increasing the protection of uninvaded areas (Peay, 2009; Gherardi et al., 2011). At present, the red swamp crayfish is considered an established exotic species in Doñana. Because its eradication is considered unfeasible, no control measures are being carried out. However, we show here that the negative impact of this invasive species is not as dramatic as in other invaded areas. Despite Doñana being one of the longest occupied European localities, the uneven and fluctuating range of this invasive crayfish species suggests that control measures taken in this area could be more successful than in other areas. Gherardi et al. (2011) reviewed the control techniques that are used against invasive crayfish. Of these, physical removal by means of fyke nets has been extensively used in the agriculturally transformed marshes surrounding the park for decades, and has failed to reduce the presence of crayfish. Predators have also been demonstrated to exert an intense negative effect on crayfish populations (Tablado et al., 2010). To increase the effectiveness of fishing and natural predation on crayfish control, it has been suggested that the surrounding agriculturally transformed marshes (rice fields with managed water fluctuations) should be allowed to experience their natural flooding regimes. This would prevent crayfish resistance in these areas and the subsequent recolonisation of natural marshes (Geiger et al., 2005; Tablado et al., 2010).

In the sandy area of the park, where the invasive crayfish's populations are unstable, crayfish can be controlled or even eradicated by intensifying physical removal methods at the few permanent sites that persist in the area during the summer, most of which are small excavated ponds. Similar programs should be applied at the few sites that retain water through the summer in the marsh.

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References

Andreu, A.C. & M. C. Villamor, 1989. Calendario reproductivo y tamaño de la puesta en el Galápago Leproso, *Mauremys leprosa* (Schweigger, 1812), en Doñana, Huelva. Doñana, ActaVertebrata 16: 167- 172.

Bernardo, J.M., A.M. Costa, S. Bruxelas & A. Teixeira, 2011. Dispersal and coexistence of two non-native crayfish species (*Pacifastacus leniusculus* and *Procambarus clarkii*) in NE Portugal over a 10-year period. Knowledge and Management of Aquatic Ecosystems (2011) 401, 28.

Blaustein, A. R., B. A. Han, R. A. Relyea, P. T. J. Johnson, J. C. Buck, S. S. Gervasi & L. B. Kats, 2011. The complexity of amphibian population declines:understanding the role of cofactors in driving amphibian losses. Annals of the New York Academy of Sciences 1223: 108-119.

Bravo, M. A., C.M. Duarte & C. Montes, 1994. Environmental factors controlling the life history of *Procamburus clarkii* (Decapoda, Cambaridae) in a temporary marsh of the Doñana National Park (SW Spain). Verhandlungen des Internationalen Verein Limnologie 25: 2450-2353.

Brooks, R.T., 2004. Weather-related effects on woodland vernal pool hydrology and hydroperiod. Wetlands 24: 104-111.

Burnham, K. P. & D.R. Anderson, 2002. Model selection and multimodel inference: a practical information-theoretic approach. Springer- Verlag, New York.

Cano, E. & M. E. Ocete, 2000. Tamaño medio y ecología reproductiva de *Procambarus clarkii* Girard (1852) (Decapoda, Cambaridae) en las marismas del bajo Guadalquivir. Zoología Baetica 11: 17-26.

Correia, A. M. & O. Ferreira, 1995. Burrowing behavior of the introduced red swamp crayfish *Procambarus clarkii* (Decapoda: Cambaridae) in Portugal. Journal of Crustacean Biology 15: 248-257.

Cruz, M. J. & R. Rebelo, 2005. Vulnerability of Southwest Iberian amphibians to an introduced crayfish, *Procambarus clarkii*. Amphibia-Reptilia 26: 293-303.

Cruz, M. J. & R. Rebelo, 2007. Colonization of freshwater habitats by an introduced crayfish, *Procambarus clarkii*, in Southwest Iberian Peninsula. Hydrobiologia 575: 191-201.

Cruz, M. J., R. Rebelo & E.G. Crespo, 2006. Effects of an introduced crayfish, *Procambarus clarkii*, on the distribution of south western Iberian amphibians in their breeding habitats. Ecography 29: 329-338.

Cruz, M. J., P. Segurado, M. Sousa & R. Rebelo, 2008. Collapse of the amphibian community of the Paul do Boquilobo Natural Reserve (central Portugal) after the arrival of the exotic American crayfish *Procambarus clarkii*. Herpetological Journal 18: 197-204.

Díaz-Paniagua, C., 1988**.** Temporal segregation in larval amphibian communities in temporary ponds at a locality in SW Spain. Amphibia-Reptilia 9: 15-26

Díaz-Paniagua, C., C. Gómez-Rodríguez, A. Portheault & W. de Vries, 2005. Los anfibios de Doñana. Serie Técnica. Ministerio Medio Ambiente-OAPN, Madrid.

Díaz-Paniagua, C., C. Gómez-Rodríguez, A. Portheault & W. de Vries, 2006. Distribución de los anfibios del Parque Nacional de Doñana en función de la densidad y abundancia de los hábitats de reproducción. Revista Española de Herpetología 20: 17-30.

Díaz-Paniagua, C., R. Fernández-Zamudio, M. Florencio, P. García-Murillo, C. Gómez-Rodríguez, A. Portheault, L. Serrano & P. Siljeström, 2010. Temporary ponds from the Doñana National Park: A system of natural habitats for the preservation of aquatic flora and fauna. Limnetica 29: 41-58.

Fahd, K., M. Florencio, C. Keller, L. Serrano, 2007. The effect of the sampling scale on zooplankton community assessment and its implications for the conservation of temporary ponds in south-west Spain. Aquatic Conservation: Marine Freshwater Ecosystems 17: 175-193.

Ficetola, G.F., M. E. Siesa, R. Manenti, L. Bottoni, F. De Bernardi & E. Padoa-Schippa, 2011. Early assesment of the impact of alien species: differential consequences of an invasive crayfish on adult and larval amphibians. Diversity Distribution 17: 1141-1151.

Florencio, M., C. Díaz-Paniagua, I. Gomez-Mestre & L. Serrano, 2011. Sampling macroinvertebrates in a temporary pond: Comparing the suitability of two techniques to detect richness, spatial segregation and diel activity. Hydrobiologia 689: 121-130.

Florencio, M., C. Díaz-Paniagua, C. Gómez-Rodriguez, C. & L. Serrano, 2013, in press.Biodiversity patterns in a macroinvertebrate community of a temporary pond network. Insect Conservation and Diversity. doi: 10.1111/icad.12029

Fortuna, M., C. Gómez-Rodríguez, J. Bascompte, 2006. Spatial network structure and amphibian persistence in stochastic environments. Proceedings of the Royal Society London B-Biological Sciences 273: 1429-1434.

Geiger, W., P. Alcorlo, A. Baltanás & C. Montes, 2005. Impact of an introduced crustacean on the trophic webs of Mediterranean Wetlands. Biological Invasions 7: 49-73.

Gherardi, F. 2007. Understanding the impact of invasive crayfish, In Gherardi, F. (ed), Biological invaders in inland waters: profiles, distribution, and threats. Invading Nature. Springer series in invasion ecology, Springer, Dordrecht; 507–542.

Gherardi, F., L. Aquiloni, J. Diéguez-Uribeondo & E. Tricarico, 2011. Managing invasive crayfish: is there a hope? Aquatic Sciences 73: 185-200.

Gomez-Mestre, I. & C. Díaz-Paniagua, 2011. Invasive predatory crayfish do not trigger inducible defences in tadpoles. Proceedings of the Royal Society B 278: 3364-3370.

Gómez-Rodríguez, C., J. Bustamante & C. Díaz-Paniagua 2010b. Evidence of hydroperiod shortening in a preserved system of temporary ponds. Remote sensing 2: 1439-1462.

Gómez-Rodríguez, C., C. Díaz-Paniagua, J. Bustamante, A. Portheault, M. Florencio, 2010a. Inter-annual variability in amphibian assemblages: Implications for diversity assessment and conservation in temporary ponds. Aquatic Conservation: Marine Freshwater Ecosystems 20: 668-677.

Gómez-Rodríguez, C., C. Díaz-Paniagua & J. Bustamante, 2011. Cartografía de Lagunas temporales del Parque Nacional de Doñana. Agencia Andaluza del Agua, Consejería Medio Ambiente, Junta de Andalucía, Sevilla.

Habsburgo-Lorena, A. S., 1978. Present situation of exotic species of crayfish introduced into Spanish continental waters. Freshwater Crayfish 4: 175-184.

Holdich, D.M., J.D. Reynolds, C. Souty-Grosset & P.J. Sibley, 2010. A review of the ever increasing threat to Euroepean crayfish from non-inidigenous crayfish species. Knowledege and Management of Aquatic Ecosystems (2009) 394-395, 11.

Hoffmann, M., Hilton-Taylor C., Angulo A.et al., 2010. The impact of conservation on the status of the world’s vertebrates. Science 330: 1503–1509.

Kats, L.B. & R.P. Ferrer, 2003. Alien predators and amphibian declines: review of two decades of science and the transition to conservation. Diversity and Distributions 9: 99-110.

Keller, C., 1998. Assessment of reproductive state *Mauremys leprosa*: a comparison between inguinal palpation and radiography. Wildlife Research 25: 527-531.

Kiesecker, J.M., 2003 Invasive species as a global problem: Toward understanding the worldwide decline of amphibians. In: Semlitsh, R.D. (ed) Amphibian conservation. Smithsonian Press,Washington, D.C.: 113-126.

Moyle, P.B. & T. Light, 1996. Biological Invasions of Freshwater: Empirical rules and assembly theory. Biological Conservation 78:149-161.

Peay, S., 2009. Invasive non-indigenous crayfish species in Europe: Recommendations on managing them. Knowledge and Management of Aquatic Ecosystems 394-395, 03:1-9.

Portheault, A., 2010. Efecto de la depredación sobre las puestas y larvas de los anfibios de Doñana. Ph. D. thesis, Universidad de Sevilla, Spain.

Portheault, A., C. Díaz-Paniagua & C. Gómez-Rodríguez, 2007. Predation on *Bufo calamita* eggs and larvae by common predators in temporary ponds. Revue Ecologie-Terre Vie 62: 315-322.

Ricciardi, A. & H.J. MacIsaac, 2011. Impacts of biological Invasions on Freshwater Ecosystems. In: Richardson, D.M. (ed), Fifty years of invasion ecology: The legacy of Charles Elton. Blackwell Publishing, Oxford: 211- 224.

Rodríguez, C. F., E. Bécares, M. Fernández-Aláez & C. Fernández-Aláez, 2005. Loss of diversity and degradation of wetlands as a result of introducing exotic crayfish. Biological Invasions 7: 75-85.

Semlitsch, R.D., 2003. Conservation of pond-breeding amphibians. In: Semlitsh, R.D. (ed) Amphibian conservation. Smithsonian Press,Washington, D.C.:8-23.

Siesa, M. E., R. Manenti, E. Padoa-Schioppa, F. De Bernardi & G. F. Ficetola, 2011. Spatial autocorrelation and the analysis of invasion processes from distribution data: A study with the crayfish *Procambarus clarkii*. Biological Invasions 13: 2147-2160.

Simberloff, D., 2005. Non-native species DO threaten the natural environment!. Journal of Agricultural and Environmental Ethics 18: 595-607.

Siljeström, P., A. Moreno, L. V. García & L. E. Clemente, 1994. Doñana National Park (south-west Spain): geomorphological characterization through a soil-vegetation study. Journal of Arid Environment 26: 315-323

Tablado, Z., J. L. Tella, J. A. Sánchez-Zapata & F. Hiraldo, 2010. The Paradox of the Long-Term Positive Effects of a North American Crayfish on a European Community of Predators. Conservation Biology 24: 1230-1238.

Valverde, J. A., 1967. Estructura de una Comunidad Mediterránea de Vertebrados Terrestres. Monografías de la Estación Biológica de Doñana, CSIC, Madrid.

Wells, K.D. 2007. The ecology and behavior of amphibians. The University of Chicago Press, Chicago and London.

Table 1. Number of different types of ponds and marsh sites sampled and crayfish percentages of occurrence over the total number of sites sampled across the whole study area and in each of the four main geomorphological areas of the park. (- indicates those areas in which a particular type of pond/site is not present)

	Total		Northern Park		Southern Park		Marsh Edge		Peridune Area	
	N sites sampled	Crayfish % occurrence	N sites sampled	Crayfish % occurrence	N sites sampled	Crayfish % occurrence	N sites sampled	Crayfish % occurrence	N sites sampled	Crayfish % occurrence
Total	405	29.1	210	28.1	100	2.0	35	45.7	18	88.9
Marshes	42	59.5	-	-	-	-	-	-	-	-
Streams	22	59.1	11	45.5	1	0	10	80.0		
Temporary ponds	265	20.8	174	23.0	62	3.2	19	26.3	10	80.0
Permanent ponds	2*	100.0	-	-	-	-	-	-	2	100.0
Excavated ponds	74	31.1	25	56.0	37	0	6	50.0	6	100.0

* These are the only permanent ponds in the study area.

Table 2. Comparison of invasive crayfish (*Procambarus clarkii*) and each amphibian species percentages of occurrence in eight temporary ponds sampled during different 2-year periods from 1978 to 2007. The differences in the amphibian percentages of occurrence across sampling periods were tested using Wilcoxon signed rank tests for paired data. After Bonferroni corrections, the significance level was set at $p < 0.0125$.

	Amphibian and crayfish percentages of occurrence in 8 temporary ponds in the northern sandy area			
	1978-1979 and 1979-1980	1983-1984 and 1984-1985	2002-2003 and 2003-2004	2005-2006 and 2006-2007
Bufo bufo	0	0	0	0
Bufo calamita	12.5	75	37.5	12.5
Pelobates cultripes	62.5	62.5	75	62.5
Discoglossus galganoi	100	12.5	37.5	12.5
Pelodytes ibericus	0	0	25	0
Pelophylax perezi	50	50	37.5	0
Hyla meridionalis	100	62.5	100	87.5
Pleurodeles waltl	75	25	100	37.5
Triturus pygmaeus	100	75	100	75
Lissotriton boscai	87.5	37.5	62.5	37.5

Procambarus clarkii	0	12.5	25	0
Test of differences with the 1978-1980 period	-	*Z*=1.153, *P*=0.249	*Z*=0.169, *P*=0.866	*Z*=2.201, *P*=0.028

Figure legends.

Figure 1. (A) Map of the Doñana National Park indicating the different areas in which crayfish distribution was examined. Northern and southern sandy areas are considered to be separated by the peridune area. The first sites at which crayfish were detected in the Doñana Biological Reserve, in 1983-1984, are indicated by stars; those first detected in 1984-1988 are indicated by white circles. (B) Crayfish presence in the park from 1991 to 2010. For sites sampled 3 or more years, permanent and occasional presences and absence are indicated. Full and empty small circles indicate data for sites sampled on a single occasion.

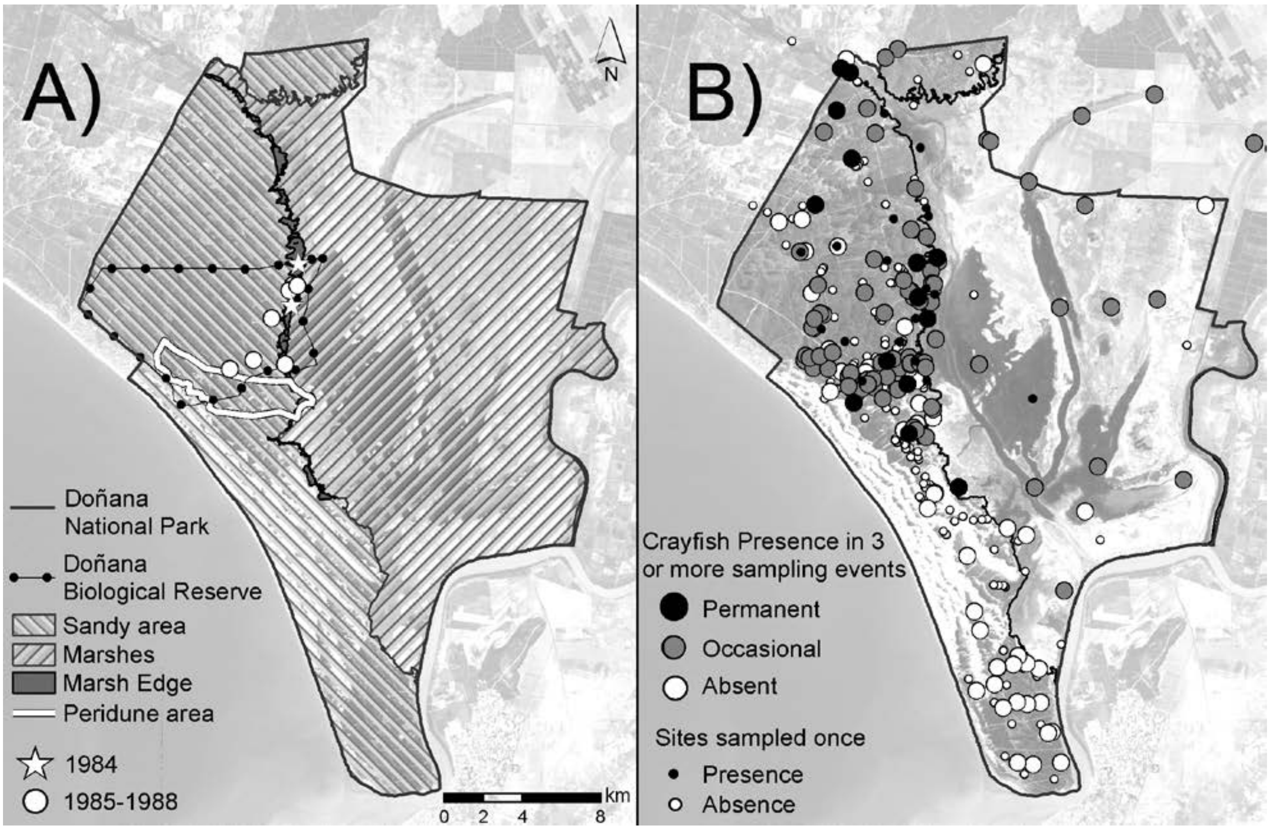
Figure 2. Variation in both annual rainfall and the percentage of red swamp crayfish occurrence (100* n sites with crayfish/ n sites sampled) from 1991 to 2010 (no data are available for 1995-96, or for 2000-2002).

Figure 3. Presence of *Procambarus clarkii* at sites sampled from 1992 to 1997. 1992 The period 1992 through 1995 were years of increasing drought. The subsequent expansion of the crayfish's range to include new sites was revealed by 1997 sampling efforts; this range expansion followed the extensive flooding that occurred in 1996, a very rainy year for which sampling data were not available. All data included in this figure were obtained with fyke nets sampling.

Figure 4. Percentages of amphibian occurrence in different areas of Doñana National Park during two periods: 2002-2004 (pre-drought) and 2005-2007 (post drought). The total amphibian percentage of occurrence has been divided to show the percentage of

sites where they co-ocurred or not with crayfish: Empty bars indicate percentage of occurrences of amphibians in the absence of crayfish, whereas black bars indicate amphibian and crayfish co-occurrence. After an extended period of drought crayfish occurrence increased at the marsh edge (b), but they disappeared from the peridune area (d). Crayfish are absent from the south of the park.

Figure 1.



A)
N
Doñana
National Park
Doñana
Biological Reserve
Sandy area
Marshes
Marsh Edge
Peridune area
1984
1985-1988
km
0 2 4 8
B)
Crayfish Presence in 3
or more sampling events
Permanent
Occasional
Absent
Sites sampled once
Presence
Absence

Figure 2.

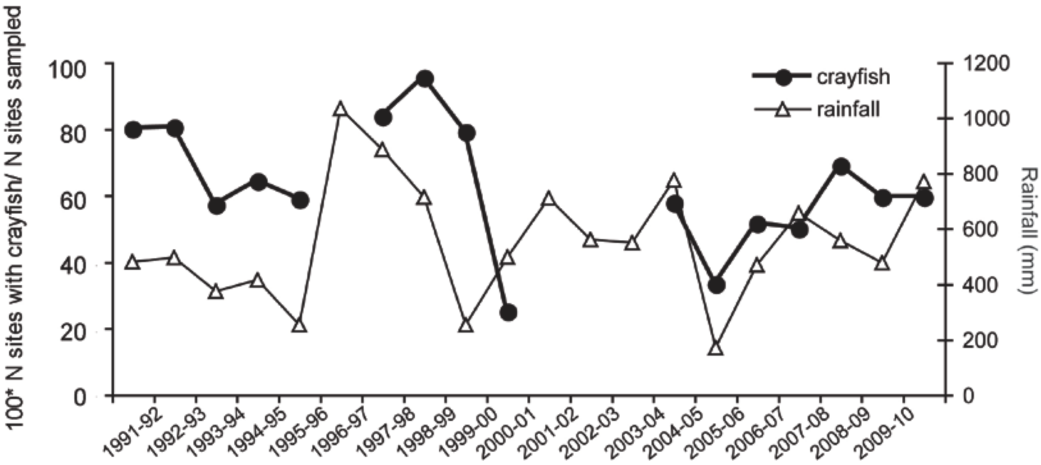

100* N sites with crayfish/ N sites sampled
100
80
60
40
20
0
crayfish
rainfall
1200
1000
800
600
400
200
0
Rainfall (mm)
1991-92
1992-93
1993-94
1994-95
1995-96
1996-97
1997-98
1998-99
1999-00
2000-01
2001-02
2002-03
2003-04
2004-05
2005-06
2006-07
2007-08
2008-09
2009-10

Figure 3.

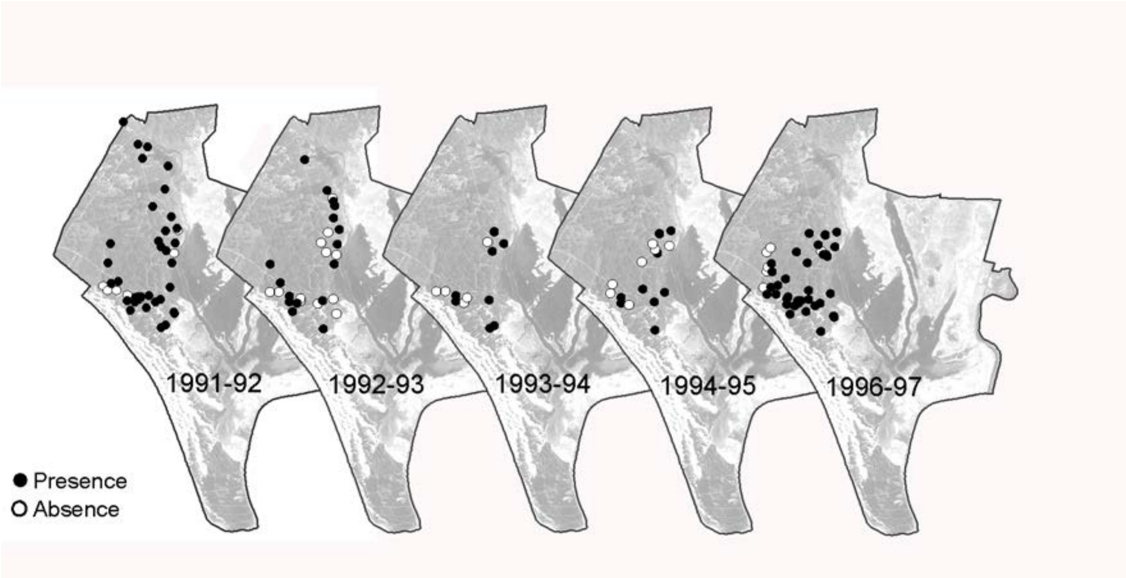
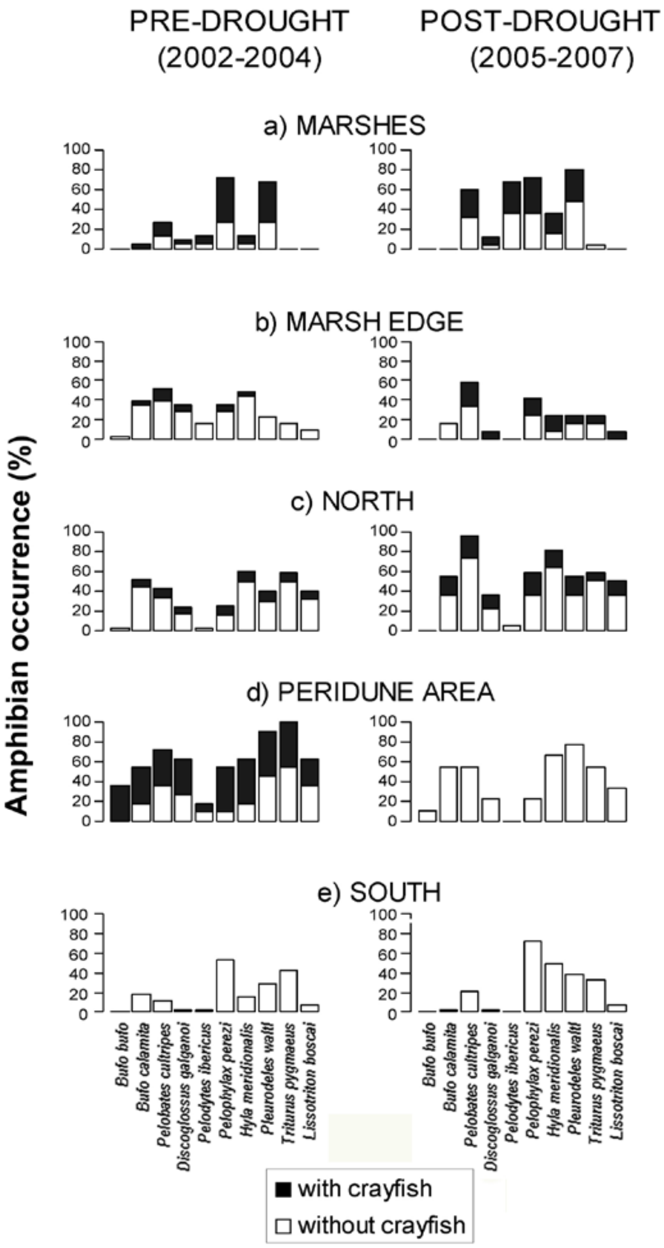


Figure 4


PRE-DROUGHT (2002-2004)
POST-DROUGHT (2005-2007)
a) MARSHES
b) MARSH EDGE
c) NORTH
d) PERIDUNE AREA
e) SOUTH
Amphibian occurrence (%)
0
20
40
60
80
100
Bufo bufo
Bufo calamita
Pelobates cultripes
Discoglossus galganoi
Pelodytes ibericus
Pelophylax perezi
Hyla meridionalis
Pleurodeles waltl
Triturus pygmaeus
Lissotriton boscai
with crayfish
without crayfish