Ecological consequences of amphibian larvae and their native and alien predators on the community structure of temporary ponds

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SUMMARY

1. Connections between consumers and resources in food webs are complex and affect the structure and functioning of ecosystems. We assessed the influence of amphibians as consumers on the structure and functioning of temporary ponds, determining their impact on macrophyte abundance, zooplankton diversity and water chemistry.

2. The effect of amphibians may be modulated by interactions with predators or competitors that alter tadpole density or behaviour. Therefore, we also investigated the consumptive and non-consumptive effects of native and invasive predators on amphibian larvae and pond ecosystems.

3. High amphibian density decreased macrophyte biomass and zooplankton diversity and increased water turbidity and nutrient content. These effects were largely attributable to tadpoles of the largest herbivorous species, spadefoot toads (Pelobates cultripes). In the absence of spadefoot toads, amphibians unexpectedly affected plant biomass positively.

4. Invasive crayfish (Procambarus clarkii) altered community structure in similar ways to high densities of amphibians and caused greater mortality of amphibian larvae than did native predators.

5. The high herbivorous impact of spadefoot toads and invasive crayfish carried over to the following hydrological cycle.

Keywords: amphibian larvae, biological invasions, community ecology, competition, red swamp crayfish, trophic web

Introduction

Extensive research has been conducted on ecological interactions within amphibian guilds, but few studies to date have explored the role of amphibian larvae in structuring aquatic communities. Fewer still have studied how larval density and trait-mediated indirect effects can modify the impact of amphibians on aquatic systems (but see Schiesari, Werner & Kling, 2009; Whiles et al., 2010; Costa & Vonesh, 2013). This is despite the fact that amphibians seem to have the potential to determine the structure and dynamics of aquatic communities (Buck et al., 2012) and to have profound and long-lasting effects on primary production, nutrient cycling, leaf litter decomposition, dynamics of invertebrate populations and energy flow between aquatic and terrestrial environments (Wilbur, 1997; Whiles et al., 2006; Regester, Whiles & Lips, 2008; Costa & Vonesh, 2013).

Amphibian larvae are common prey for a suite of vertebrate and invertebrate predators, and predation can also alter the outcome of the ecological interactions of their prey (Morin, 1986). However, amphibians respond to predators by activating adaptive plastic changes in their morphology, behaviour and/or timing of ontogenetic switch points (Anholt & Werner, 1995; Relyea, 2001; Van Buskirk, 2001). Such plastic responses increase amphibian survival (Lima, 1998; Van Buskirk & McCallum, 2000) but often result in reduced activity and lower growth rates (Skelly, 1997; Miner et al., 2005), suggesting altered consumption rates on trophic resources such as periphyton, zooplankton and macrophytes (e.g. Costa & Vonesh, 2013). Different types of predators, however,
may have different consumptive and non-consumptive effects, and native predators are more likely to exert non-consumptive effects than invasive predators. Activation of appropriate plastic defences depends critically on the accurate detection of predator cues, and local prey may fail to recognise invasive predator cues and hence fail to activate their defences against them (Polo-Cavia et al., 2010; Gomez-Mestre & Diaz-Paniagua, 2011). This lack of innate recognition of invasive predators relates to a lack of joint evolutionary history between local prey and novel predators, and it may confer a high-disruptive potential on invasive predators.

We tested experimentally the effects of amphibian density and guild composition on the structure and dynamics of Mediterranean temporary ponds. Using an array of mesocosms, we assessed the effect of amphibians on macrophyte diversity and biomass, zooplankton abundance, zooplankton abundance and diversity, and water chemistry. Macrophytes constitute an essential element in aquatic food webs, both because they are a source of energy for many herbivores, including tadpoles, but also because they provide shelter for zooplankton and a substratum for periphyton development, and decrease water turbidity by reducing sediment suspension (Van Donk & Van de Bund, 2002). Zooplankton abundance and composition is also a determining factor for primary production and is strongly dependent upon water turbidity and the presence of organisms at higher trophic levels (Carpenter et al., 1987; Des Roches et al., 2013). We expected amphibians to change water physicochemistry and the composition of zooplankton and to decrease macrophyte biomass through direct herbivory. We studied the effect on community structure of the local amphibian guild as a whole, but also tested the effect of western spadefoot toad (Pelobates cultripes) tadpoles specifically, because these are much larger and have a longer larval period than the other amphibians we investigated (Díaz-Paniagua et al., 2005). Consequently, we expected a disproportionately greater effect on community structure of spadefoot toad tadpoles than the rest of the anuran guild. Furthermore, we studied the role of native and invasive predators in modifying the effect of the anuran guild by comparing both consumptive and non-consumptive effects of dytiscid beetle larvae (Dytiscus circumflexus: native predators) and red swamp crayfish (Procambarus clarkii: invasive predators). We experimentally distinguished non-consumptive predator effects (derived from caged predators altering tadpole behaviour and/or morphology) from the combination of non-consumptive and consumptive effects (from free predators reducing tadpole density), which could alter tadpole trophic ecology (Caut et al., 2013) and hence their role in the aquatic food web. We expected uncaged invasive and native predators to reduce amphibian survival, hence allowing survivors to grow bigger. Direct tadpole consumption would reduce the net effect of amphibian larvae on community structure. We expected crayfish to have a bigger effect on community structure than dytiscid beetle larvae, because crayfish are larger predators, local tadpoles do not seem to recognise their chemical cues, and they are omnivores with a potentially high direct impact on macrophyte biomass. We expected caged native predators fed on tadpoles to provide predator cues and tadpole alarm cues in the experimental ponds and thus to exert non-consumptive effects on tadpoles via behaviour modification. However, given the lack of innate recognition of alien crayfish cues, we did not expect such non-consumptive effects from caged red swamp crayfish.

Methods

Study system

The study was carried out in Doñana National Park (37°00′N, 6°38′W) in south-west Spain on the right bank of the Guadalquivir River mouth. This area contains more than 1100 temporary ponds in a 6794 ha area (Díaz-Paniagua et al., 2010), which are usually flooded in autumn or winter (Díaz-Paniagua et al., 2010). Most amphibian species in the area use temporary ponds as breeding habitats (Díaz-Paniagua et al., 2005). A total of 11 amphibian species are found in the Park. In our study, we included the six species most commonly found in temporary ponds (Díaz-Paniagua, 1990) to have a representative characterisation of the amphibian larval guild of the area. We included five anuran species: the western spadefoot toad, the Mediterranean treefrog (Hyla meridionalis), the Iberian green frog (Pelophylax perezi), the Iberian painted frog (Discoglossus galganoi) and the natterjack toad (Bufo calamita). We also included a urodele, the pygmy marbled newt (Triturus pygmaeus), which is very abundant in Doñana and whose larval diet is largely comprised of planktonic crustaceans (93%; Díaz-Paniagua et al., 2005). A more detailed description of each species is given in Appendix SI in Supporting Information.

As native predators, we used dytiscid beetle larvae (Dytiscus circumflexus), which occur in temporary ponds of the Park. We used red swamp crayfish as an invasive predator and a potential competitor for amphibians. Red swamp crayfish were introduced in the neighbourhood
of Doñana National Park in 1974 (Habsburgo-Lorena, 1978) and have since expanded into many aquatic habitats throughout the Park. The crayfish are voracious predators of amphibian eggs and larvae (Cruz & Rebelo, 2005; Portheault, Díaz-Paniagua & Gómez-Rodriguez, 2007) and also exert strong effects as primary consumers of macrophytes and as filter-feeders (Gutiérrez-Yurrita, 1998; Cronin et al., 2002). Thus, red swamp crayfish are not just tadpole predators (Cruz & Rebelo, 2005; Ficetola et al., 2011), and they are also competitors that use many of the same resources that tadpoles consume (mostly macrophytes and algae; Caut et al., 2013).

Experimental array

We established an experimental array of mesocosms at Doñana Biological Reserve consisting of 8 treatments: (1) no amphibian larvae (No Amph): absence of amphibians or their predators; (2) low amphibian density (Low): presence of low density of larvae of six amphibian species; (3) high density of amphibian larvae (High): a three-fold increase with respect to initial density in the low treatment; (4) absence of P. cultripes (No Pc): as in low treatment but excluding Pelobates cultripes, the largest species with the longest developmental period; (5) caged native predator (NatC): low density of amphibian larvae exposed to one caged Dytiscus larva; (6) free native predator (NatF): low density of amphibian larvae exposed to a single free ranging Dytiscus larva; (7) caged invasive predator (InvC): low density of amphibian larvae exposed to one caged red swamp crayfish; and (8) free invasive predator (InvF): low density of amphibian larvae exposed to a single free ranging red swamp crayfish. We considered low density to be three individuals per tank for P. cultripes, T. pygmaeus, D. galganoi and P. perezi, and 10 individuals per tank for H. meridionalis. Tadpole densities fell well within the range commonly observed during field surveys in the Park (Díaz-Paniagua, 1990). Each treatment was replicated 12 times for a total of 96 tanks distributed in 12 randomised complete blocks.

The experiment lasted for 10 weeks between 24 March and 2 June 2011. Mesocosms consisted of 500-L-rounded plastic tanks, 100 cm high and 120 cm in the upper diameter. To replicate the conditions of the natural ponds in Doñana, we added 105.6 ± 0.18 kg from the basin of a dry temporary pond where several amphibian species usually breed and an extra 1.125 kg of an evenly combined mixture of sediment from nine other temporary ponds in the area. Mesocosms were filled with 250 L of well water, and we added a 5 L inoculum of pond water collected from two temporary ponds that contained zooplankton and phytoplankton. Furthermore, we planted several species of aquatic macrophytes to provide spatial complexity and as a food base for the aquatic community. Thus, we added in each tank 25–30 stems of Myriophyllum alterniflorum, 8–10 stems of Ranunculus peltatus, 40–50 stems of Callitriche obtusangula and four stems of Mentha pulegium. These species provide a good representation of the macrophytes present in the temporary ponds of Doñana (Díaz-Paniagua et al., 2010).

The tanks were covered with fibreglass window screens that were opened for several hours a day to allow colonisation by flying insects. Each tank also contained a floating, lidded 1-L plastic bucket with holes drilled in the bottom, where predators were held in tanks assigned to caged predator treatments.

Introduction and removal of amphibians and predators

We collected the six species of amphibian larvae by dip-netting in a number of different temporary ponds within the Doñana Biological Reserve. Larvae of each amphibian species were haphazardly assigned to each experimental tank from a species pool of field-collected larvae. We added larvae to the tanks following the natural breeding phenology of each species (Díaz-Paniagua, 1988): on 24 March, we introduced P. cultripes and B. calamita; on 29 and 30 March, we introduced H. meridionalis and T. pygmaeus; on 8 and 9 April, we introduced D. galganoi, and finally on 17 May, we introduced P. perezi. All anuran larvae were between 25 and 30 Gosner stages (Gosner, 1960), and newt larvae were between 40 and 44 stages (according to the developmental stages of Pleurodeles waltl in Shi & Boucaup, 1995). We also collected predators (crayfish and dytiscid larvae) from ponds within the Reserve and haphazardly allocated them to either caged or free predator treatments. Because predator and alarm cues may degrade after c. 48 h (Peacor, 2006), we fed each caged predator two amphibian larvae every 3 days throughout the experiment, randomly drawing from one of the six amphibian species used for this study, to ensure a constant source of the appropriate cues. Tadpoles fed to caged predators were kept in additional outdoor tanks prepared in the same way as the experimental tanks.
We checked the tanks twice daily for metamorphs (i.e. Gosner stage 42) from the time we observed the first metamorph until the end of the experiment. We digitally photographed every group of amphibian larvae and every free or caged predator entering each tank at the beginning and at the end of the experiment. We also measured larval body length and total length, snout-to-vent length (SVL) of metamorphs and predator body length with ImageJ 1.46r (NIH, U.S.A.). At the end of the experiment, we also recorded body mass of all metamorphs and remaining larvae. We also recorded the date of forelimb emergence (i.e. Gosner stage 42; Gosner, 1960). Nonetheless, our design precluded comparisons of larval period across species, because different species were incorporated into the experiment at different times, according to their breeding phenology.

**Physicochemical parameters**

We recorded pH, dissolved oxygen and electrical conductivity with a YSI 6600V2-4 multiprobe four times during the course of the experiment. We also recorded turbidity at the end of the experiment with a turbidimeter. We took a 330 mL water sample from every tank at the end of the experiment on 30 May to determine the level of chlorophyll-a (Chl-a, μg L⁻¹, tricromatic method; Holmansen & Riemann, 1978) and the concentration of four dissolved nutrients in the water (ammonium, phosphate, nitrite and nitrate). Nutrient concentrations were determined with a multi-channel Seal Analytical AutoAnalyzer, Norderstedt, Germany. Water samples were filtered through Whatman GF/F 47-mm glass microfibre filters 0.7 μm in pore size, using a low-pressure vacuum pump. Filtered water was bottled and frozen until analysed. Filters were kept for chlorophyll-a analysis.

**Sample collection**

At the end of the experiment (30–31 May), we collected zooplankton samples by filtering 5 L of water from the water column from each tank through a 100-μm plankton net. We preserved the samples in a 70% ethanol to quantify the zooplankton species abundance. We identified the species in our samples under an inverted microscope using published taxonomic accounts of aquatic invertebrates (Galindo et al., 1994).

At the end of the experiment, we harvested all plants remaining in the tanks. We removed the excess water with a manual centrifuge and recorded the fresh weight of every plant species per tank: Myriophyllum alterniflorum, Ranunculus peltatus, Callitriche obtusangula and two species of charophytes. To test for carry-over effects of experimental treatments on plant biomass onto the next hydrological cycle, we let the tanks dry naturally over the summer and then refilled them in the autumn with well water to mimic pond re-flooding. Plants grew naturally from seed in each tank after re-flooding and were allowed to grow for 7 weeks. We then harvested all plants in each tank, separated them by species and weighed them.

**Data analysis**

We excluded two tanks from all data analyses (one replicate belonging to the native caged predator treatment and one to low density treatment) due to extremely low outlier values in plant biomass as high numbers of Triops mauritanicus emerged from the sediment in these tanks and strongly affected water quality and depleted macrophyte biomass from the beginning of the experiment. No B. calamita survived in the experiment and only nine metamorphs of D. galganoi were observed emerging throughout the experiment, mostly in the treatment without P. cultripes. Experimental tanks may not constitute ideal habitats for these two species that usually breed in shallow areas of short-lasting ponds; despite the basking ramp we had explicitly added. Thus, differences among treatments in survival and size at metamorphosis were not estimated for these species.

We used generalised linear mixed models (GLMM) to test for the effect of experimental treatments on our response variables using the GLIMMIX procedure (Schaubenberger, 2007) in SAS. 9.2 (SAS-Institute, Cary, NC, U.S.A.). For all dependent variables, we calculated the mean within each experimental tank and used tank means for statistical analyses. In the case of survival, we modelled the number of survivors per tank out of the initial number of individuals. We tested the effect of experimental block as a random factor and removed it from the model when it was not significant.

Response variables were transformed to meet parametric assumptions as follows: total plant biomass and Myriophyllum biomass were squared-root-transformed, whereas phosphate and chlorophyll concentrations were log-transformed; we used the inverse for all other nutrients and conductivity, and we ranked the variable turbidity. Morphological measurements were normally distributed and homoscedastic for all species (except for P. perezi body mass, which we square-root-transformed). We found no among-treatment differences in initial size of any of the species studied, but initial size had a significant effect on survival of H. meridionalis and P. perezi.
We therefore included initial size as a covariate in analyses of survival.

Normally distributed variables were modelled with a Gaussian error distribution and an identity link function. The biomasses of three plants (Ranunculus, Callitrichie and Chara) were analysed using integers, and we fitted models using a negative binomial distribution and a log link function. We modelled amphibian survival assuming an underlying binomial error distribution and a logit link function, including initial size as a covariate to control for any possible effect of species differences in size. Since our experimental design was not fully factorial, we specified multiple contrasts through planned comparisons to test specific hypotheses. We therefore tested for effects of presence/absence of amphibians and the effect of increasing their density with the following contrasts: No Amph-Low, No Amph-High and Low-High; we tested the effect of P. cultripes comparing Low-No Pc; and we tested the effect of predators with that of the amphibian presence at low density (low-predators) and with different combinations of native and invasive caged and free predators (Table 1). Given that several of these multiple comparisons (15 comparisons total) were not orthogonal, we corrected the observed P-values to minimise the false discovery rate (FDR; Benjamini & Hochberg, 1995; García, 2003).

We calculated the abundance of each zooplankton species per litre of water and also estimated the diversity of aquatic invertebrates in each sample. We estimated the Shannon-Wiener diversity index using the package VEGAN within R (R Core Development Team, Vienna, Austria).

We also conducted a multivariate analysis with PRIMER-E (Clarke & Gorley, 2006) including the physicochemical variables in addition to plant biomass at the end of the experiment. We failed to collect physicochemical data from eight tanks and these had to be discarded in the multivariate analysis since the software requires full matrices. The variables were log-transformed and squared-root-transformed and normalised to obtain a dissimilarity matrix using Euclidean distance for environmental variables prior to performing a non-metric multidimensional scaling (NMDS) ordination (Clarke & Gorley, 2006). An NMDS ordination was also performed with the four main groups of zooplankton (cladocerans, copepods, rotifers and ostracods) after log-transforming the data and standardising the samples by dividing by total counts to produce a resemblance matrix using the Bray Curtis similarity index.

Table 1 Contrasts performed to test for effects of amphibian larvae and their predators on temporary ponds. Effects of amphibians are tested by its presence/absence, Low/No Amph; increased density, High; or exclusion of Pelobates cultripes, No Pc. We also used the contrasts shown on the right to test the effects of predators on the amphibian guild and ultimately on the trophic web.

<table>
<thead>
<tr>
<th>Contrasts</th>
<th>Effects of amphibians on temporary ponds</th>
<th>Effects of predators on amphibians and on temporary ponds</th>
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<tr>
<td>No Amph – Low</td>
<td>Low – NatC</td>
<td>Low – NatF</td>
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<td>No Amph – High</td>
<td>Low – InvC</td>
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<tr>
<td>Low – High</td>
<td>NatC – NatF</td>
<td>InvC – InvF</td>
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<tr>
<td>No Amph – No Pc</td>
<td>NatC – InvC</td>
<td>NatC – InvF</td>
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<td>Low – No Pc</td>
<td>NatF – InvC</td>
<td>NatF – InvF</td>
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NatC, caged native predator; NatF, free native predator; InvC, caged invasive predator; InvF, free invasive predator.

Results

Effects of amphibians in temporary ponds

Amphibian larvae had a considerable effect on water physicochemistry (Fig. 1; see Tables S1 & S2 in Supporting Information). Turbidity increased 3.7-fold in the presence of amphibians at low density (No Amph-Low; F,186 = 10.85; P = 0.0024) and 6.4-fold at high amphibian density (No Amph-High; F,186 = 20.58; P < 0.001). When P. cultripes was absent, however, turbidity was not different from tanks without amphibians (Fig. 1a). Amphibian larvae decreased oxygen concentration by 15.9% at low density (No Amph-Low; F,175 = 10.91; P = 0.001) and 26.2% at high density (No Amph-High; F,175 = 27.38; P < 0.001; Fig. 1c). Amphibians increased ammonium concentration by more than twofold at both densities, although these were not significant (No Amph-Low; F,178 = 2.89; P = 0.09; Fig. 1e). In the presence of amphibians, we also observed increased water conductivity in low (F,186 = 5.95; P = 0.031) and high larval density (F,186 = 19.42; P < 0.001; Fig. 1b). We observed a marked effect of P. cultripes on water chemistry (Low-No Pc; Fig. 1): tanks with amphibian larvae but lacking P. cultripes had on average 18% more dissolved oxygen (F,175 = 13.07; P < 0.001), 62% less ammonium (F,178 = 6.24; P = 0.0029) and 45% less phosphate (F,178 = 8.07; P = 0.017) than those with P. cultripes.

We observed a significant increase in chlorophyll-a concentration, an indirect measure of phytoplankton biomass, when the amphibian guild was at high density.
compared with tanks lacking amphibian larvae \((F_{1,78} = 4.84; P = 0.0308)\), whereas no significant increase was detected at low amphibian density or when \(P. cultripes\) was absent (Fig. 2a; Table S1).

Regarding the aquatic vegetation, by the end of the experiment, there was a 50% less plant biomass in tanks with a low density of amphibian larvae than in tanks without amphibians \((F_{1,86} = 14.95; P < 0.001)\) and a 85% less plant biomass at high amphibian density \((F_{1,86} = 64.84; P < 0.001;\) Fig. 2b). Two of the macrophyte species, \(C. obtusangula\) and \(R. peltatus\), were completely consumed in treatments containing amphibian larvae, whereas \(M. alterniflorum\) was never depleted. Charophyte biomass did not vary across treatments. When \(P. cultripes\) was excluded but all other amphibians were present, macrophyte biomass actually increased by an average of 45% compared with tanks without amphibians \((F_{1,86} = 4.42; P = 0.038;\) Fig. 2b).

The presence of amphibian larvae reduced zooplankton diversity by an average of 12% when at low density, although the effect was only significant when amphibian density was high, reducing zooplankton diversity by 25% on average \((F_{1,86} = 9.63; P = 0.003;\) Tables S2 & S3 in Supporting Information). Thus, the zooplankton changed in composition at high amphibian density, increasing its copepod abundance (mainly due to a greater number of nauplii and copepod larvae; No Amph-High; \(F_{1,85} = 19.98; P < 0.001)\), whereas cladoceran abundance was reduced (No Amph-High; \(F_{1,85} = 15.03; P < 0.001)\) and ostracods were almost absent (No Amph-High; \(F_{1,85} = 11.44; P = 0.004;\) Fig. 3a). The presence of the amphibian guild also increased the abundance of copepods (No Amph-Low; \(F_{1,85} = 4.71; P = 0.033)\). At low amphibian density, we also observed a trend towards decreased numbers of cladocerans, although the effect was non-significant (No Amph-Low; \(F_{1,85} = 2.58; P = 0.112)\). In the absence of \(P. cultripes\), however, zooplankton composition was similar between treatments with and without amphibians. Rotifers were not significantly affected by any of the treatments, although there was a trend of increased density in the absence of \(P. cultripes\) (No Amph-No PC; \(F_{1,85} = 2.94; P = 0.09)\; Fig. 3a).

Because the different amphibian species were added to the tanks sequentially to mimic reproductive phenology, among-species comparisons of survival, weight at metamorphosis and time to metamorphosis could not be made. Overall, amphibian survival at low density was 43.6 and 31.6% at high larval density (Low-High; \(F_{1,75} = 10.87; P = 0.002)\), whereas it increased to 53% when \(P. cultripes\) was excluded (Low-No PC; \(F_{1,75} = 4.01; P = 0.047\).
P = 0.049; Fig. 4a). At low density, survival of *H. meridionalis* increased when *P. cultripes* was excluded (Low-No Pc; $F_{1,74} = 8.17; P = 0.009; \text{Fig. 4c}$), but high density reduced its survival (Low-High; $F_{1,74} = 41.51; P < 0.001$).

Although not statistically significant, the same trend was observed for *P. perezi* (Fig. 4c). *Pelobates cultripes* and *T. pygmaeus* had high survival in all treatments without predators (Fig. 4b,e).

**Fig. 2** (a) Variation in chlorophyll-\(a\), an indirect measure of phytoplankton. (b) Biomass of the three most abundant macrophytes plus charophytes remaining in each treatment at the end of the experiment and (c) after re-flooding the tanks. Bars represent the contribution of each species to the final biomass, and error bars represent the standard error of the total plant biomass.

The species varied in the extent to which they had completed larval development by the end of the experiment. Thus, most *H. meridionalis* individuals had completed metamorphosis (90% of survivors), whereas 44.4% of *P. cultripes* and only 3 and 5%, respectively, of *T. pygmaeus* and *P. perezi* had completed metamorphosis (Fig. 4). *Pelobates cultripes* had the lowest proportion of metamorphs at high density, and those that metamorphosed emerged at a smaller body size (18.3% reduction in SVL; $F_{1,48} = 27.6; P < 0.001; \text{Fig. 4b}$) and lower body mass (52% reduction; $F_{1,49} = 43.86; P < 0.001$) than at low density.

In addition to the negative effects of *P. cultripes* on survival of other amphibian species, we also found that 44% of *D. galganoi* larvae that reached metamorphosis emerged at a smaller body size (18.3% reduction in SVL; $F_{1,48} = 27.6; P < 0.001$; Fig. 4b) and lower body mass (52% reduction; $F_{1,49} = 43.86; P < 0.001$) than at low density.

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Effects of native predators on amphibian larvae and pond communities

The presence of native predators (dytiscid larvae) had no effects on water chemistry, phytoplankton, aquatic vegetation or zooplankton diversity and composition (Figs 1, 2 & 3; Tables S1 & S3 in Supporting Information). The presence of free dytiscid larvae reduced overall amphibian survival from 43.6 to 34.85% (NatC-NatF; $F_{1,75} = 8.87; P = 0.005$; Fig. 4a). However, *P. cultripes* metamorphs were 6.5% longer ($F_{1,49} = 4.27; P = 0.018$) and 18.5% heavier ($F_{1,49} = 7.89; P = 0.018$) in the presence of free native dytiscid larvae than when dytiscid larvae were caged. There was also a marginally significant increase in *H. meridionalis* size at metamorphosis ($F_{1,68} = 3.92; P = 0.052$; Fig. 4c).

Effects of the red swamp crayfish on amphibian larvae and pond communities

The red swamp crayfish affected most parameters (see Low-InvF comparisons in Figs 1, 2 & 3; Tables S1 & S3 in Supporting Information). The presence of free dytiscid larvae reduced overall amphibian survival from 43.6 to 34.85% (NatC-NatF; $F_{1,75} = 8.87; P = 0.005$; Fig. 4a). However, *P. cultripes* metamorphs were 6.5% longer ($F_{1,49} = 4.27; P = 0.018$) and 18.5% heavier ($F_{1,49} = 7.89; P = 0.018$) in the presence of free native dytiscid larvae than when dytiscid larvae were caged. There was also a marginally significant increase in *H. meridionalis* size at metamorphosis ($F_{1,68} = 3.92; P = 0.052$; Fig. 4c).

![Fig. 4](image_url) (a) Overall survival for all five species included in this study. (b–e) Metamorphic and larval survival across treatments for the various amphibian species. Bars indicate overall survival across treatments (+SE) considering both life stages, each column partitioned to indicate the proportion of individuals remaining as larvae (in grey) or metamorphs (in black). Metamorphic snout-vent length is only provided for *H. meridionalis*, as this was the only species for which most individuals had metamorphosed by the end of the experiment (mean ± SE).

We found a non-significant trend towards increased chlorophyll-a in the presence of free crayfish (Fig. 2a). Tanks containing free crayfish had practically no plants at the end of the experiment, as plant biomass was reduced by 95% compared with the absence of predator treatment (Low-InvF; $F_{1,86} = 54.94; P < 0.001$; Fig. 2b).

Zooplankton diversity did not vary among treatments with and without predators (see data in Table S1). However, copepods were more abundant when crayfish were free than when they were caged ($F_{1,85} = 11.08; P = 0.003$). Ostracods were not abundant in either treatment with crayfish, but their abundance was lower in tanks with freely roaming crayfish (InvC-InvF; $F_{1,85} = 3.96; P = 0.049$; Fig. 3a).

Free crayfish reduced amphibian survival from 43.6 to 20.4% (Low-InvF; $F_{1,75} = 29.74; P < 0.001$; Fig. 4a). *Pelobates cultripes* experienced the highest overall survival rate and only suffered high mortality when predators...
were free, especially crayfish (Low-InvF; $F_{1,63} = 23.66; P < 0.001$; Fig. 4b). Crayfish reduced survival in *H. meridionalis* by 32% (InvC-InvF; $F_{1,74} = 3.89; P = 0.052$; Fig. 4c) and by 50% in *P. perezi* (InvC-InvF; $F_{1,74} = 5.8; P = 0.018$; Fig. 4d), and caused complete mortality of *T. pygmaeus* (Fig. 4e).

*Pelobates cultripes* tadpoles remaining at the end of the experiment in tanks with free-roaming crayfish had smaller SVL (16% reduction; Low-InvF; $F_{1,38} = 8.52; P = 0.015$) and mass (34% reduction; Low-InvF; $F_{1,38} = 5.89; P = 0.025$).

**Persisting effects over the next hydrological cycle**

The effect of amphibian larvae on plant biomass persisted in the next hydrological cycle, after tanks dried up and were re-flooded in the autumn, without adding any more amphibians. Tanks that had contained amphibians at low density during the experiment had c. 60% less plant biomass 7 weeks after re-flooding than tanks that had not contained amphibians (No Amph-Low; $F_{1,75} = 25.34; P < 0.001$). Amphibian density also had a carry-over effect, as tanks that had contained high density of amphibians had c. 75% less plant biomass after re-flooding than tanks that had contained no amphibians (No Amph – High; $F_{1,75} = 47.27; P < 0.001$; Fig. 2c). The impact of free-roaming crayfish on plant biomass also persisted after re-flooding, as plant biomass was 67.7% lower in tanks that had contained free crayfish than tanks that had not (Low-InvFree; $F_{1,75} = 11.5; P = 0.001$; Table S1 & S3 in Supporting Information). The carry-over effect of amphibians and crayfish was evident for *M. alterniflorum* (Fig. 2c), whereas biomasses of *R. peltatus* and *C. obtusangula* only varied between low amphibian density and absence of *P. cultripes*, increasing in the absence of *P. cultripes*.

**Combined effects of amphibians and predators on community structure**

Non-metric multidimensional scaling on turbidity, nutrients, oxygen, chlorophyll-α and plant biomass at the end of the experiment provided an overall view of the effects of the experimental treatments (Fig. 5). NMDS grouped tanks that did not contain amphibians with tanks that contained amphibians but not *P. cultripes* (Fig. 5). Tanks in these two treatments (No Amphibians and No *P. cultripes*) had a higher oxygen concentration, lower turbidity and chlorophyll-α and greater plant biomass. At the other end of the gradient, tanks containing a high density of amphibians and tanks containing free-roaming crayfish were also grouped, determined by their higher nutrient content (except for nitrates) and higher conductivity and turbidity. Tanks with caged predators or only amphibians in low density were located between these two extremes. Likewise, NMDS grouped tanks according to zooplankton composition so that tanks with high amphibian density showed a higher abundance of copepods and rotifers, whereas tanks with no amphibians or with amphibians but excluding *P. cultripes* had a predominance of cladocerans and abundant ostracods, similar to tanks with free native predators. Tanks with free invasive crayfish, however, were in an intermediate position with similar abundances of copepods and cladocerans, as in the case of tanks with low amphibian density or with caged predators (Fig. 3b).

**Discussion**

**Effects of amphibians on temporary ponds**

Our results show that amphibian larvae can have an important impact as key primary consumers on the structure of freshwater communities and that individual amphibian species may differ in the magnitude of their effects and even exert opposite effects. In this study, amphibians affected pond water quality, algal abundance, plant biomass and zooplankton diversity. These effects were magnified at high amphibian density, which also caused almost complete depletion of aquatic vegetation, directly or indirectly induced algal proliferation (as indicated by increased chlorophyll-α content) and increased water turbidity and nutrient content, and decreased oxygen.

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Nutrients and grazing interact to control periphyton productivity and growth of submersed macrophytes (Marks & Lowe, 1989). Water turbidity affects nutrient dynamics, in particular of nitrate and ammonium, which can in turn produce algal blooms that shade aquatic macrophytes and reduce carbon exchange at leaf surfaces, constraining their growth or even causing their death (e.g. Sand-Jensen & Borum, 1991). Amphibians also markedly reduce macrophyte biomass. Macrophytes constitute food resources for tadpoles and play essential roles in freshwater ecosystems because they increase water clarity and the complexity of the physical habitat (Scheffer et al., 1993, 2001), critical for amphibian survival. The sharp decrease in plant biomass, however, could not be attributable to all tadpole species, but was largely caused by those of spadefoot toads. The reduction in macrophyte biomass carried over to the next hydrological cycle, as tanks that had contained high amphibian density during the experiment had substantially fewer plants growing in them upon refilling in the autumn. Hence, macrophytes may have been consumed prior to flowering, fruits may have been damaged, seed production may have been reduced by plants under tadpole herbivory, or several of these factors may have combined to result in reduced plant biomass after re-flooding.

In the absence of *P. cultripes*, however, the rest of the amphibians had a positive effect on macrophyte abundance, directly or indirectly. To the best of our knowledge, this is a novel and unexpected effect of amphibians on community structure. It is known that grazers such as isopods, amphipods and gastropods, together with nutrient enrichment, have a strong positive effect on macrophyte production (Neckles, Wetzel & Orth, 1993). Kupferberg (1997) found that tadpole grazing removed epiphytes that shaded filamentous green algae and transformed them into excreted nutrients, hence facilitating microalgae growth and productivity, although this effect differed according to tadpole species. The reduced turbidity, increased oxygen concentration, and decreased ammonium and phosphate in the water in tanks without *P. cultripes* can also be indirect factors benefitting aquatic macrophytes, or conversely these factors could result from abundant healthy macrophytes.

Of all the species comprising the amphibian guild in Doñana, *P. cultripes* clearly stands out in terms of its disproportionate contribution to the observed amphibian-driven alterations of the aquatic environment. Tadpoles of *P. cultripes* are much bigger than the other species in our system, breed early in the season and have the longest larval period (Díaz-Paniagua et al., 2005), which also makes them unrivalled competitors in Mediterranean guilds (Richter-Boix, Llorente & Montori, 2007). Large herbivorous tadpoles thus have the potential to alter the life history and possibly the population dynamics of aquatic plants. Large tadpoles can forage disproportionately more not only due to allometric differences in size-dependent filtering ability and consumption rates (Wassersug, 1975), but also because the smaller tadpoles are displaced and have reduced access to valuable resources (Richter-Boix, Llorente & Montori, 2004).

The presence of amphibian larvae also affected zooplankton composition. Urodele larvae can decrease zooplankton biomass and affect its density and composition (Holomuzki, Collins & Brunkow, 1994). In our study, the reduced zooplankton diversity observed at high amphibian density could have direct and indirect causes. Direct predation by newt larvae is likely to have been the primary factor in reducing zooplankton diversity. Additionally, anuran larvae, as primary consumers, could also have exerted resource competition over zooplanktonic species for algae (Mokany, 2007), contributing to reduce its diversity, especially at high density. Moreover, the decrease in macrophyte biomass and consumption of preferred phytoplankton may also have favoured the proliferation of other algae (Kupferberg, 1997). In line with this idea, we found increased chlorophyll-a at high amphibian density, which, together with the increase in turbidity and detritus, may have influenced the shift in zooplankton composition from cladoceran to copepod dominance.

Effects of predators on amphibian larvae and on community structure

Free dytiscid larvae greatly reduced amphibian survival, mostly affecting *P. cultripes* and *H. meridionalis*. By reducing the number of individuals, predators release surviving larvae from competition resulting in their increased size (Morin, 1983, 1986), as we detected in surviving *P. cultripes* and *H. meridionalis* metamorphic individuals. However, predator consumption did not reduce the impact of amphibian larvae on community structure, and non-consumptive effects were almost negligible in this study.

Invasive red swamp crayfish had a large impact on the environment and directly caused changes in water quality, nutrient content and macrophyte biomass. Red swamp crayfish strongly affect various trophic levels in freshwater systems, via both consumptive and
non-consumptive processes (e.g. macrophyte cutting or uprooting; Anastácio, Parente & Correia, 2005; Gherardi & Acquistapace, 2007). In our study, the presence of freely roaming crayfish in the tanks increased ammonium, phosphate and nitrite in accordance with previous studies (Angeler et al., 2001). This release of nutrients into the water may be produced through bioturbation (Angeler et al., 2001) or excretion (Evans-White & Lambert, 2005). Increased nutrient levels could be caused by feeding of caged predators (Costa & Vonesh, 2013), but that effect was not found in our study. On the other hand, Matsuzaki et al. (2008) found that crayfish reduced the concentration of dissolved ammonium probably due to its rapid uptake by phytoplankton, showing the complexity of possible trophic connections within freshwater systems. We also observed increased copepod abundance in the presence of freely roaming crayfish. Another study suggested that crayfish may have positive effects on zooplankton biomass via changes in phytoplankton abundance (Dorn & Wojdak, 2004), but we found no evidence for crayfish effects on algal abundance.

The negative impact of invasive crayfish on amphibian survival was much stronger than the overall impact of native predators. Red swamp crayfish are much bigger than any other invertebrate in the area and native amphibians lack a joint evolutionary history with it. Hence, amphibian larvae from Doñana cannot recognise the cues from this invasive species and consequently fail to trigger inducible anti-predator defences against them (Gomez-Mestre & Díaz-Paniagua, 2011). This double role of invasive crayfish as competitors and generalist predators renders them very harmful in aquatic communities (Ficetola et al., 2012), with the potential to markedly reduce local amphibian densities (Cruz & Rebelo, 2005; Cruz, Rebelo & Crespo, 2006).

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Brief description of every amphibian species included in this experiment: *Pelobates cultripes*, *Triturus pygmaeus*, *Discoglossus galganoi*, *Bufo calamita*, *Hyla meridionalis* and *Pelophylax perezi*.

Table S1 Mean ± SE of the chemical parameters measured in tanks at the end of the experiment per treatment: turbidity, chlorophyll-α, Shannon Diversity Index for the zooplankton, total final plant biomass and after re-flooding the tanks, dissolved oxygen, conductivity and nutrients.

Table S2 Statistical results of the GLMM to test the effect of amphibians on different ecological variables of temporary ponds.

Table S3 Statistical results of the GLMM to test the effect of native and invasive predators on different ecological variables of temporary ponds.

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