

Early assessment of the impact of alien species: differential consequences of an invasive crayfish on adult and larval amphibians

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ABSTRACT

Aim Early assessment of the impact of invasive alien species is crucial to set up timely management, but often the impact is evident when it is too late for action. We evaluated relationships between the alien crayfish, *Procambarus clarkii*, the distribution of native amphibians, and the abundance of their larvae. We assessed whether considering measures of reproductive success provide a more prompt measure of impact than considering just species distribution.

Location One hundred and twenty-five wetlands in Northern Italy, in an area recently invaded by *P. clarkii*.

Methods We surveyed wetlands to assess the presence of breeding activity of amphibians and the distribution of *P. clarkii*. We measured the abundance of amphibian larvae before metamorphosis through pipe sampling. We built models analysing the relationships between amphibian and crayfish distribution, while taking into account spatial autocorrelation and environmental features. Analyses were performed at both the species level (generalized linear models and spatial eigenvector mapping) and community level (constrained redundancy analysis).

Results In terms of breeding site distribution, only two amphibians (*Lissotriton vulgaris* and *Hyla intermedia*) were negatively associated with *P. clarkii*, while the relationships between other the species and *P. clarkii* were positive or not significant. However, larval abundance for all amphibian species was negatively associated with the alien crayfish. Analyses performed at community and single species levels yielded consistent results.

Main conclusions *Procambarus clarkii* impacts amphibians through different processes. Newts probably avoid invaded wetlands for breeding. Other species attempt breeding in wetlands with crayfish, but suffer very low success. Considering distribution data alone would not provide a correct picture of the impact of this alien species; measures of reproductive success may allow a more accurate assessment of the impact.

Keywords

Amphibian decline, biological invasions, breeding habitat, breeding success, landscape composition, *Procambarus clarkii*, spatial autocorrelation.

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INTRODUCTION

Invasive alien species (IAS) are a major cause of biodiversity loss at the global scale. Management of IAS has a higher likelihood of success if performed at early stages of invasions (i.e., immediately after introduction, or soon after naturalization)

(Hulme, 2006). Nevertheless, only a subset of species that are introduced become invasive and cause negative consequences (Jeschke & Strayer, 2005; Vila *et al.*, 2010). Therefore, management often focuses on those species with the strongest impact on biodiversity, or in the areas where these species determine the worst consequences (Vila *et al.*, 2010). Rapid identification

of the effects of IAS on native biota is a necessary first step for prompt management actions. However, an early assessment of the effects of IAS may be complex. In the long term, negative consequences of IAS are expected to affect the distribution of native species, thus observations of decline or range contraction are common measures of impact. Species distribution data and time series provide key information to assess whether IAS are causing declines (e.g., Strayer *et al.*, 2006; Cruz *et al.*, 2008; Elliott *et al.*, 2010). However, considerable time may pass before negative effects of IAS are revealed in distribution data, depending on generation time and site fidelity. Signs of stress may become apparent only after it is too late for effective management. Measuring the impact of IAS on fitness parameters of native species may allow a more prompt evaluation of processes ongoing, and of the actual consequences of IAS. The abundance of juveniles may be an important proxy of fitness, even though relationships between abundance of juveniles and fitness may be complex and nonlinear, because of density-dependent effects (e.g., intraspecific competition; Vonesh & De la Cruz, 2002).

Complex relationships between environmental features and species distribution constitute a further issue to understanding the impact of IAS. Environmental modifications, such as urbanization or the degradation of natural environments, often have negative consequences on native species. At the same time, introductions are more frequent in human-dominated areas, and human-induced modifications facilitate the expansion of some IAS (Didham *et al.*, 2007). As both native and IAS are influenced by the same environmental features, it may be difficult to establish whether the decline of a species was caused by IAS or by environmental modifications. Analyses considering both habitat modifications and IAS are needed to tease apart their relative role. Nevertheless, only a small subset of studies consider the effects of IAS jointly to the ones of environmental modifications (Didham *et al.*, 2007).

The American red swamp crayfish, *Procambarus clarkii*, is native of eastern North America and Mexico, but has been introduced world-wide and is currently invasive in wide areas of Europe (Gherardi & Panov, 2009; Capinha & Anastácio, 2011). *Procambarus clarkii* is a keystone IAS that can have multiple consequences on both native species and ecosystem functioning. For instance, *P. clarkii* can cause the decline of native crayfishes through competition and the spread of the crayfish plague, *Aphanomyces astaci* (Gherardi, 2006; Gherardi & Panov, 2009), it preys on aquatic stages of amphibians and can cause the decline of several amphibian species (Gherardi *et al.*, 2001; Kats & Ferrer, 2003; Riley *et al.*, 2005; Cruz *et al.*, 2006a,b, 2008; Witte *et al.*, 2008), and can reduce macrophytes determining the shift of aquatic ecosystems from a clear water phase, with abundant submerged vegetation, to a turbid phase, without macrophytes (Matsuzaki *et al.*, 2009).

In this study, we evaluated the relationships between *P. clarkii* and native amphibians. We compared analyses of the distribution of breeding adults with analyses of the distribution of larvae, to assess whether considering different life-history stages can provide different measures of the impact

of the crayfish on native species. We considered an area of Northern Italy where the crayfish has been introduced about 5 years ago (Fea *et al.*, 2006) and where it still has a limited distribution (see Results). This stage may represent the phase at which managers decide whether control efforts are required. First, we evaluated the relationship between the distribution of *P. clarkii* and the distribution of amphibian breeding wetlands, while taking into account potential effects of environmental features on species distribution. Subsequently, we analysed the relationship between *P. clarkii* and the abundance of larvae, as a measure of breeding success in wetlands where we observed breeding activity. We show that the two analyses offer a different perspective on the consequences of *P. clarkii* on native amphibians, and that the analysis of breeding success may allow a more prompt and appropriate assessment of the impact on some native species. Each analysis was performed at two levels: single species and whole community. Single species analyses help to identify more precisely the situation of target species, and community analyses allow unravelling more complex and general relationships.

METHODS

Study area and methods outline

We studied 125 wetlands (slow stream ditches, ponds and small lakes) in Lombardy, Northern Italy. The study area (lat: 45.4–45.9°N; long: 8.8–9.4°E) comprises the upper portion of the Po river lowland and the Brianza foothills (altitude 150–397 m). See Ficetola *et al.* (2011b) for a map of the study area. The landscape is dominated by agricultural and urban areas. Nevertheless, several natural parks protect important sites for amphibian conservation (e.g., Ficetola *et al.*, 2009). First, we surveyed all the wetlands to assess relationships between the distribution of amphibian breeding sites and *P. clarkii*. Subsequently, we evaluated the abundance of amphibian larvae in a subset of wetlands where amphibians bred, and we tested whether *P. clarkii* causes a loss of breeding success in these wetlands. The occurrence of spatial autocorrelation increases the complexity of analysing these relationships. The distribution of both native and IAS can be strongly affected by spatial autocorrelation: nearby localities can have similar occupancy because species distribution is limited by dispersal mechanisms and because species are influenced by environmental features (e.g., vegetation, climate, landscape composition) which tend to be spatially autocorrelated (Beale *et al.*, 2010). Therefore, we integrated spatial autocorrelation into all our analyses.

Distribution of breeding sites

We surveyed each wetland four to six times from March to June: 114 wetlands were surveyed in 2009, 11 further wetlands were surveyed in 2010. During each survey, we used multiple techniques to assess the presence of breeding amphibians and *P. clarkii*. For amphibians, we used visual encounter surveys, clutch counts, and repeated dip netting of wetland bottom and

banks. We also performed audio point counts (5 min each) to identify calling males (Dodd, 2010). For *P. clarkii*, we used nocturnal visual surveys, dip netting and the identification of exuviae (Souty-Grosset *et al.*, 2006). We performed at least three night surveys and at least one day survey. We also recorded four survey-specific environmental variables: air temperature, air humidity (%), wind speed (Beaufort scale) and presence of rain during the survey.

Abundance of larvae

In late May–early June 2010, we used pipe sampling to quantitatively assess the abundance of amphibian larvae and *P. clarkii* in 34 ponds (Dodd, 2010). In this period, larvae of all amphibian species are still in the breeding wetlands. Samples were collected by thrusting the pipe (a 0.25-m² circular sampler) through the water column and into the sediments, to seal the sample area. Small nets were used to remove all animals from the water column and the first centimetre of the sediment. Net sweeps were collected until at least 10 consecutive empty sweeps (Werner *et al.*, 2009; Dodd, 2010). Number of pipe samples was proportional to wetland surface (average: four samples per wetland). In most wetlands, we collected four to six samples, but a smaller number were collected in a few, small wetlands. We used detectability analyses to assess the reliability of this sampling. To maximize the possibility to detect relationships between amphibians and *P. clarkii*, pipe sampling was focused on a non-random subsample of sites. We sampled sites where we detected reproductive activity of amphibians, while keeping a balanced ratio (50%) between wetlands invaded and not invaded by the crayfish. In all the sampled sites, at least one species of brown frog (*Rana latastei* or *R. dalmatina*) laid eggs. Furthermore, we sampled most wetlands where we detected newts. Larvae were identified following standard keys (Lanza *et al.*, 2007). Tadpoles of *R. dalmatina* and *R. latastei* are morphologically similar, making field identification uncertain (Barbieri *et al.*, 2000). Therefore, we pooled tadpoles of these two species into a single group ('brown frogs') for some analyses. For each species, we calculated abundance as number of larvae m⁻². Individuals of *P. clarkii* were weighed, to measure their mass (g m⁻²).

Environmental variables

In late May–June, we characterized amphibian breeding wetlands using eight parameters: maximum width, maximum depth, average stream velocity, canopy cover and percentage of riparian vegetation along the shoreline, percentage of emerging or floating vegetation on wetland surface, fish presence and water permanence (Table 1). Parameters were recorded using standard bio-assessment protocols; details of their measurement are reported elsewhere (Barbour *et al.*, 1999; Van Buskirk, 2005; Ficetola *et al.*, 2011a; see Table 1). Furthermore, we used the ARCVIEW GIS (© ESRI, 1999) to measure the land cover in the landscape surrounding each wetland, on the basis of the 2008 vector map of the Lombardy region ([**Table 1** Environmental variables recorded. Some groups of variables have been summarized by factors extracted through principal component analyses \(PCA\) prior to perform single species or community analyses.](http://</p>
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Environmental features	Summarized by PCA?
Wetland features	
Surface* (m)	Abiotic features
Maximum depth* (cm)	Abiotic features
Permanence during the study period (Y/N)	Abiotic features
Stream velocity* (m s ⁻¹)	
Fish presence (Y/N, visual estimate)	
Canopy cover (% , visual estimate)†	
Emerging vegetation (% of vegetation emerging from water surface, visual estimate)†	
Riparian vegetation (% , visual estimate)†	
Landscape features	
Forest cover (%)†‡	Landscape 400
Cropland cover (%)†‡	Landscape 400
Urban cover (%)†‡	Landscape 400

*Log-transformed prior to analysis.

†Square-root arcsin-transformed prior to analysis.

‡Measured in a radius of 400 m.

www.cartografia.regione.lombardia.it). We considered three land cover typologies (Table 1): urban, agriculture and natural vegetation. Land cover was measured in radius of 400 m from each wetland (Ficetola *et al.*, 2009).

Statistical analyses

Species abundance obtained through pipe sampling and several environmental variables were not normally distributed, and were transformed to reduce skewness and improve normality (see Table 1). Some measures of wetland morphology (area, depth and hydroperiod) and of landscape composition were strongly correlated (Pearson's correlation: $|r|$ ranging from 0.3 to 0.8). To remove multicollinearity, and to limit the number of candidate models, we used principal component analyses (PCA) to reduce correlated variables to a smaller number of uncorrelated factors. A first PCA run over the variables describing abiotic features (Table 1), extracted one variable explaining 51% of variance. This variable (hereafter named abiotic features) was positively associated with area ($r = 0.62$), depth ($r = 0.83$) and hydroperiod ($r = 0.65$). Therefore, high values of abiotic features indicate large, deep and permanent wetlands. Similarly, we ran PCA over the three variables describing landscape composition (Table 1). The component extracted (landscape 400) explained 63% of variation and was positively associated with urban ($r = 0.76$) and agricultural cover ($r = 0.58$), and negatively associated with natural vegetation ($r = -0.99$) at this scale. We ran PCA separately for abiotic features and landscape for a better interpretation of resulting scores. We found no strong correlation among environmental variables or PCA scores used for analyses ($|r| \leq 0.4$ in all correlations).

Detectability analysis

A site is surely ‘occupied’ if a species is detected at that site, but non-detecting a species during all sampling occasions does not necessarily indicate that the species is absent. This can lead to an underestimation of occupancy and might affect the results of analyses. We used PRESENCE 2.4 (Hines, 2006) to estimate occupancy and evaluate the probability of occupancy of sites where we did not detect species (MacKenzie *et al.*, 2006; Sewell *et al.*, 2010). For the breeding site data set, we assumed that the probability of detection of species at a given survey might be affected by six survey-specific covariates: Julian date, hour of survey, air temperature, cloud cover, wind speed and humidity %. For each species, we built models assuming that detection probability depends on all possible combinations of these covariates; we considered the model with the lowest Akaike’s information criterion (AIC) as the minimum adequate model describing species detectability (Burnham & Anderson, 2002). We calculated misdetection rate as the percentage difference between the observed occupancy and the occupancy estimated from the PRESENCE model; we assumed that a species was reliably detected if misdetection rate was < 5%. For a few species, misdetection rate was > 5% (see Results). For these species, we re-ran species models, by using the probability of occupancy at a given site (as estimated by PRESENCE) as dependent variable. Models were run separately for sites surveyed in different years. For the pipe sampling data set, we ran occupancy models assuming that probability of detection (presence/absence) of larvae of each species was constant across all the samples collected in a given site.

Species distribution

We analysed the relationship between the distribution of breeding sites of each species, the environmental features and the distribution of *P. clarkii*. We used an information theoretic approach, based on AIC (Burnham & Anderson, 2002), to identify the combination of variables best describing the distribution of amphibians detected in > 10% of sites. We built generalized linear models (GLM) assuming binomial error, including all possible combinations of environmental variables. For each model, we calculated AIC and Δ -AIC, which is the difference in AIC between a candidate and the model with lowest AIC (i.e., the best model). The use of AIC as sole selection criterion may select overly complex models; therefore, we used two additional criteria. We considered a complex model only if it had a Δ -AIC lower than the Δ -AIC of all its simpler nested models; furthermore, we considered only models including significant variables (Raffalovich *et al.*, 2008; Denoël *et al.*, 2009; Richards *et al.*, 2011). For each candidate model, we also calculated Nagelkerke’s R^2 (R^2_N) as a measure of variance explained. Per each model i , we calculated the AIC weight w_i , which is the probability for a model to be the best one among the candidates (Richards *et al.*, 2011). Furthermore, we compared the best models with similar models not considering the presence of *P. clarkii*. Spatial autocorrelation may bias results of

standard regression techniques; therefore, if residuals were autocorrelated, we used spatial eigenvector mapping (SEVM) instead of standard GLM. SEVM allows the translation of the spatial arrangement of data points into explanatory variables capturing the spatial effects. We identified the eigenvector(s) best reducing spatial autocorrelation, and we included them into the models (Moran’s eigenvectors; see Dormann *et al.*, 2007; Peres-Neto & Legendre, 2010 for details). This implementation of SEVM is considered among the most robust spatial methods (Dormann *et al.*, 2007).

We used constrained redundancy analysis (RDA) to assess the relationship between the composition of amphibian communities, environmental features and *P. clarkii*. RDA is a canonical analysis that allows to evaluate how much of the variation of the structure of a multivariate data set (e.g., species composition) is explained by one or more data sets representing independent variables (e.g., environmental variables) (Legendre & Legendre, 1998). Spatial autocorrelation may affect also canonical analyses; therefore, we integrated autocorrelation in RDA. We identified the Moran’s eigenvectors reducing spatial autocorrelation of each species, while controlling for the effect of environmental variables. The matrix including all the eigenvectors selected for at least one species was identified as the spatial matrix (Peres-Neto & Legendre, 2010). To assess the relationship between amphibian communities and environmental features, we considered community composition as dependent, environmental features as constraining matrix (i.e., independent) and the spatial matrix as conditioning matrix (the effect of which is partialled out). Similarly, to assess the relationship between amphibians and *P. clarkii*, we considered the presence of *P. clarkii* as constraining matrix, and both the environmental and spatial matrices as conditioning. We calculated the significance of variance explained by RDAs by performing permutation tests (10,000 permutations) (Legendre & Legendre, 1998).

Larval abundance

We used GLMs assuming normal error distribution to assess the relationships between the abundance of amphibian larvae and *P. clarkii*. First, we built models relating larval abundance to environmental variables. In these models (‘environment-only’), we included the environmental variables selected by the analysis of species distribution. Subsequently, we built models considering both environmental features and the presence of *P. clarkii* (‘environment + *P. clarkii*’), and compared models with and without *P. clarkii* using AIC and significance testing. If residuals of GLMs were spatially autocorrelated, we used SEVM instead of standard GLM. We considered the mass of *P. clarkii* (g m^{-2}) as a measure of its abundance. We built models for all species detected in > 15% of sites. One of these species (*Triturus carnifex*) was not analysed with species distribution model. For this species, the ‘environment-only’ model included a constant only. Analyses including combinations of potential environmental variables yielded identical results.

Subsequently, we used a series of RDAs to assess the relationships between larval communities (abundance of all species), environmental features and abundance of *P. clarkii*, while taking into account the effect of spatial autocorrelation. We performed all analyses using packages *SPDEF* and *VEGAN* within the R statistical environment (<http://www.r-project.org>).

RESULTS

We detected *P. clarkii* in 19 wetlands (occupancy, $O = 15\%$), and the presence of nine amphibians: fire salamander *Salamandra salamandra* ($O = 10\%$), Italian crested newt *Triturus carnifex* ($O = 8\%$), smooth newt *Lissotriton vulgaris* ($O = 12\%$), common toad *Bufo bufo* ($O = 17\%$), Italian tree frog *Hyla intermedia* ($O = 22\%$), agile frog *Rana dalmatina* ($O = 40\%$), Italian agile frog *R. latastei* ($O = 37\%$), common frog *R. temporaria* ($O = 4\%$) and pool frog *Pelophylax synklepton esculentus* ($O = 56\%$). Four species (Italian crested newt, Italian tree frog, agile frog and Italian agile frog) are included in the annexes II and/or IV of the 'habitat' directive of the European Union (EC 43/1992) and require strict protection of populations and their habitats. For the majority of species, misdetection rate was $< 5\%$. Misdetection rate was $> 5\%$ for three species (crested newt: misdetection $M = 8\%$, tree frog: $M = 26\%$ and pool frog: $M = 7\%$) indicating that these species might have remained undetected at some sites.

Table 2 Relationships between species distribution, environmental features and presence of *Procambarus clarkii*: comparison of models with and without the effect of *P. clarkii*.

Species	Model including <i>P. clarkii</i>							Without <i>P. clarkii</i>		
	Sign	χ^2	P	R^2_N	K	AIC	w	K	AIC	w
<i>Lissotriton vulgaris</i>										
<i>P. clarki</i>	–	14.2	< 0.001	0.62	4	53.4	0.998	3	65.7	0.002
Abiotic features	+	19.2	< 0.001							
Fish	–	5.8	0.016							
SEVM eigenvectors		32.1	< 0.001							
<i>Hyla intermedia</i>										
(a)										
<i>P. clarki</i>	–	4.1	0.042	0.38	4	107.0	0.533	3	109.1	0.186
Landscape 400	+	4.1	0.043							
Canopy cover	–	7.5	0.006							
SEVM eigenvectors		20.4	< 0.001							
(b)										
<i>P. clarki</i>	–	4.9	0.027	0.37	4	108.7	0.228	3	111.6	0.053
Abiotic features	+	5.7	0.017							
Landscape 400	+	5.5	0.019							
SEVM eigenvectors		25.7	< 0.001							
<i>Pelophylax s. esculentus</i>										
<i>P. clarki</i>	+	4.8	0.029	0.18	3	161.8	0.800	2	164.5	0.200
Abiotic features	+	6.0	0.014							
Landscape 400	+	8.3	0.004							

The table includes species for which the presence of *P. clarkii* was included in the best AIC models. Spatial eigenvector mapping (SEVM) was used for *L. vulgaris* and *H. intermedia*. R^2_N , Nagelkerke's R^2 ; K , number of parameters in the model; AIC, Akaike's information criterion; w , AIC weight.

Species distribution: single species analyses

For three species (smooth newt, tree frog and pool frog), the best AIC models included the presence of *P. clarkii* (Table 2). For these species, we compared models with and without *P. clarkii*. The smooth newt was associated with relatively large, non-ephemeral wetlands without fish and without crayfish. The pool frog was associated with relatively large, permanent wetlands within human-dominated landscapes, and with presence of crayfish (Table 2). For the tree frog, two slightly different models had similar support. Both models indicate that the tree frog was associated with wetlands without crayfish within human-dominated landscapes. Furthermore, one model suggested that this species was associated with sunny wetlands, a second model suggested association with relatively large wetlands (Table 2).

For the smooth newt, the support of the model without crayfish was very low, indicating that distribution is most likely negatively affected by crayfish presence. Conversely, for the pool frog and the tree frog, the support of models not considering crayfish presence was > 0.18 , indicating some uncertainty in the identification of the 'best' model and of the effect of crayfish (Table 2).

Crayfish presence was not included in any candidate model for the common toad, the agile frog and the Italian agile frog (Table 3). The common toad was associated with wetlands within natural landscapes, with fish and abundant riparian vegetation. The agile frog was associated with abundant

Species	Variables	Best AIC models							Effect of <i>P. clarkii</i>	
		Sign	χ^2	<i>P</i>	R^2_N	<i>K</i>	AIC	<i>w</i>	χ^2_1	<i>P</i>
<i>Bufo bufo</i>	Landscape 400	–	24.0	< 0.001	0.56	4	72.4	1	0.2	0.644
	Emerging vegetation	+	14.0	< 0.001						
	Fish	+	12.0	< 0.001						
	SEVM eigenvector		11.9	< 0.001						
<i>Rana dalmatina</i>	Riparian vegetation	+	19.6	< 0.001	0.51	3	116.9	1	2.7	0.104
	SEVM eigenvectors		33.0	< 0.001						
<i>Rana latastei</i>	(a)									
	Abiotic features	+	24.8	< 0.001	0.44	5	128.1	0.52	1.6	0.204
	Landscape 400	–	5.9	0.015						
	Canopy cover	+	7.5	0.006						
	Emerging vegetation	+	20.8	< 0.001						
	Riparian vegetation	+	17.8	< 0.001						
	(b)									
	Landscape 400	–	25.6	< 0.001	0.44	5	128.3	0.48	2.2	0.138
	Canopy cover	+	6.9	0.009						
	Emerging vegetation	+	22.0	< 0.001						
	Riparian vegetation	+	18.4	< 0.001						
	Fish	+	5.8	0.016						

The table includes species for which the presence of *P. clarkii* was not included in the best models and reports the significance of *P. clarkii*, if added to the best models. Spatial eigenvector mapping (SEVM) was used for *B. bufo* and *R. dalmatina*. See Table 2 for abbreviations.

riparian vegetation (Table 3). For the Italian agile frog, two models had similar support. Both models indicate that this frog was associated with shaded wetlands, with abundant riparian and aquatic vegetation, within the most natural landscapes. Furthermore, one model suggested that this frog was associated with relatively large, permanent wetlands, while the second model suggested association with wetlands occupied by fish (Table 3).

The best AIC models explained a substantial percentage of variation for the smooth newt, the common toad, the agile frog and the Italian agile frog, while the amount of explained variation was lower for the tree frog and the pool frog (Tables 2 and 3). The SEVM eigenvectors, representing spatial autocorrelation, were incorporated and explained a significant amount of variation in the models of the smooth newt, tree frog, common toad and agile frog (Tables 2 and 3).

For tree frog and pool frog, detectability analyses suggested some degree of imperfect detection. Therefore, we repeated analyses using occupancy estimated by PRESENCE as dependent variable (see Table S1 in Supporting Information). The best models were similar to the standard models presented in Table 2, but tended to include a lower number of variables and explained a lower amount of variation. For tree frog, the presence of *P. clarkii* was not included in any of the best models (Table S1).

Species distribution: community analysis

Redundancy analysis showed that after controlling for the effect of spatial autocorrelation, community structure was

Table 3 Relationships between species distribution, environmental features and presence of *Procambarus clarkii*.

strongly and significantly affected by environmental features (permutation test, $P < 0.0001$; Fig. 1a). Relationships between amphibians and environmental features were similar to the ones depicted by univariate models (Table 2, Fig. 1a). After taking into account the effect of spatial autocorrelation and environmental features, the relationship between amphibian communities and the alien crayfish was significant (permutation test, $P < 0.0001$; Fig. 1b). The pool frog, agile frog and Italian agile frog were associated with wetlands invaded by the crayfish. Conversely newts, salamander, common toad and tree frog were associated with wetlands without crayfish (Fig. 1b). Variance partitioning showed that environmental variables accounted for 39% of explained variation in community structure, spatial autocorrelation accounted for 40%, while crayfish presence accounted for 6%. The remaining variation was explained by the joint effects of multiple variables.

The analysis performed using probability of occupancy, estimated by PRESENCE, as dependent variable yielded identical results (effect of environmental features: $P < 0.0001$; effect of *P. clarkii*: $P = 0.0007$; Fig. S1 in Supporting Information).

Distribution of larvae

We detected *P. clarkii* in 50% of wetlands surveyed through pipe sampling. Furthermore, we detected larvae of seven amphibian taxa: *S. salamandra* ($O = 9\%$), *T. carnifex* ($O = 35\%$), *L. vulgaris* ($O = 44\%$), *B. bufo* ($O = 3\%$), *H. intermedia* ($O = 17\%$), *P. s. esculentus* ($O = 21\%$) and brown frogs (i.e., *R. dalmatina* + *R. latastei*) ($O = 79\%$). Detectability analysis

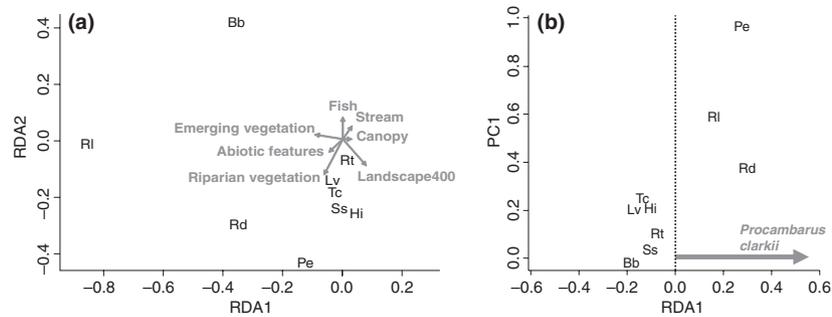


Figure 1 Results of constrained redundancy analysis showing (a) the relationship between wetland features and amphibian distribution, while taking into account spatial autocorrelation; (b) the relationship between amphibian distribution and presence of *Procamburus clarkii*, while taking into account wetland features and autocorrelation. Ss, *Salamandra salamandra*; Tc, *Triturus carnifex*; Lv, *Lissotriton vulgaris*; Hi, *Hyla intermedia*; Bb, *Bufo bufo*; Rd, *Rana dalmatina*; Ri, *R. latastei*; Pe, *Pelophylax s. esculentus*. Constraining variables are in grey colour.

showed that for all species, misdetection rate of larvae was < 3%.

Univariate models showed that after taking into account the effect of environmental variables, the abundance of larvae was negatively associated with the abundance of crayfish (Fig. 2, Table 4). For all species, the models taking into account the abundance of alien crayfish had much higher support than the models without crayfish. Models considering environmental features only explained a limited amount of variation, while in most of cases models considering also the abundance of crayfish explained a substantial amount of variation (Table 4). Residual autocorrelation was significant for the larvae of the smooth newt only. For this species, one SEVM eigenvector was incorporated into the models (Table 4). As the juveniles of *P. clarkii* tend to include more food of animal origin in their diet than adults (Correia, 2003), we repeated analyses using the number of individuals m^{-2} as a measure of crayfish abundance. Also in these analyses, for all species, larval abundance was negatively related to crayfish abundance (Table S2).

The relationship between larval abundance and the alien crayfish was negative and significant also for pool frogs (Table 4, Fig. 2d), despite breeding sites surveys indicated a positive association between crayfish distribution and this species (Table 2). This suggests that pool frogs often attempt breeding in wetlands with crayfish, but breeding is not successful. To test this hypothesis, we repeated the analysis considering only the 23 wetlands where we detected pool frog breeding activity and we performed pipe sampling. In these wetlands, the abundance of pool frog larvae was negatively associated with the abundance of *P. clarkii* ($F_{1,19} = 4.9$, $P = 0.039$; regression model taking into account landscape and abiotic features). *Procamburus clarkii* was present in 39% of these 23 wetlands.

Redundancy analysis showed that after taking into account spatial autocorrelation and environmental features, the relationship between larval communities and the alien crayfish was significant (permutation test, $P = 0.012$). For all species, the abundance of larvae was negatively associated with crayfish abundance. Environmental variables accounted for 9% of explained variation in community structure, autocorrelation accounted for 25%, while crayfish presence accounted for 18%

of explained variation. The remaining variation was explained by the joint effects of multiple variables.

DISCUSSION

We observed strong relationships between the invasive crayfish *P. clarkii* and amphibian communities, but the estimates of crayfish impact, based on species distribution data, were different from those obtained through analysis of the abundance of larvae (Figs 1b & 2). The analysis of species distribution data was based on the presence of breeding adults and egg masses and suggested that only some species were negatively associated with *P. clarkii* (i.e., did not breed in invaded wetlands), while others (pool frog and brown frogs) often breed in invaded wetlands (Tables 2 and 3, Fig. 1b). Observing such a result, without considering data on breeding success, would suggest the incorrect conclusion that these frogs are not negatively affected by the alien crayfish. The analysis of larvae lead to very different conclusions: for all species, the abundance of larvae was negatively associated with the distribution of the alien crayfish, suggesting an impact on reproductive success (Table 4, Fig. 2). Therefore, the alien crayfish may affect amphibian communities through multiple mechanisms: loss of suitable breeding sites and loss of fitness if breeding occurs in invaded sites.

Some species, such as newts, may avoid breeding in invaded wetlands because of the predation pressure (Cruz *et al.*, 2006b). For instance, newts have a complex breeding behaviour; males may defend territories for courtship and both sexes remain in breeding wetlands for relatively long periods (Lanza *et al.*, 2007). Therefore, adults may directly experience predation by crayfish and avoid invaded wetlands. Native species may also avoid invaded sites because IAS alter environmental features and make them unsuitable (Didham *et al.*, 2007). For instance, *P. clarkii* can reduce the presence of macrophytes in waterbodies (Matsuzaki *et al.*, 2009). Some amphibians select wetlands with abundant aquatic vegetation, which provides support for deposition and shelter for larvae (e.g., Cruz *et al.*, 2006b; Lanza *et al.*, 2007). In these cases, *P. clarkii* may negatively impact amphibians through the loss of breeding wetlands. In human-dominated landscapes, the number of

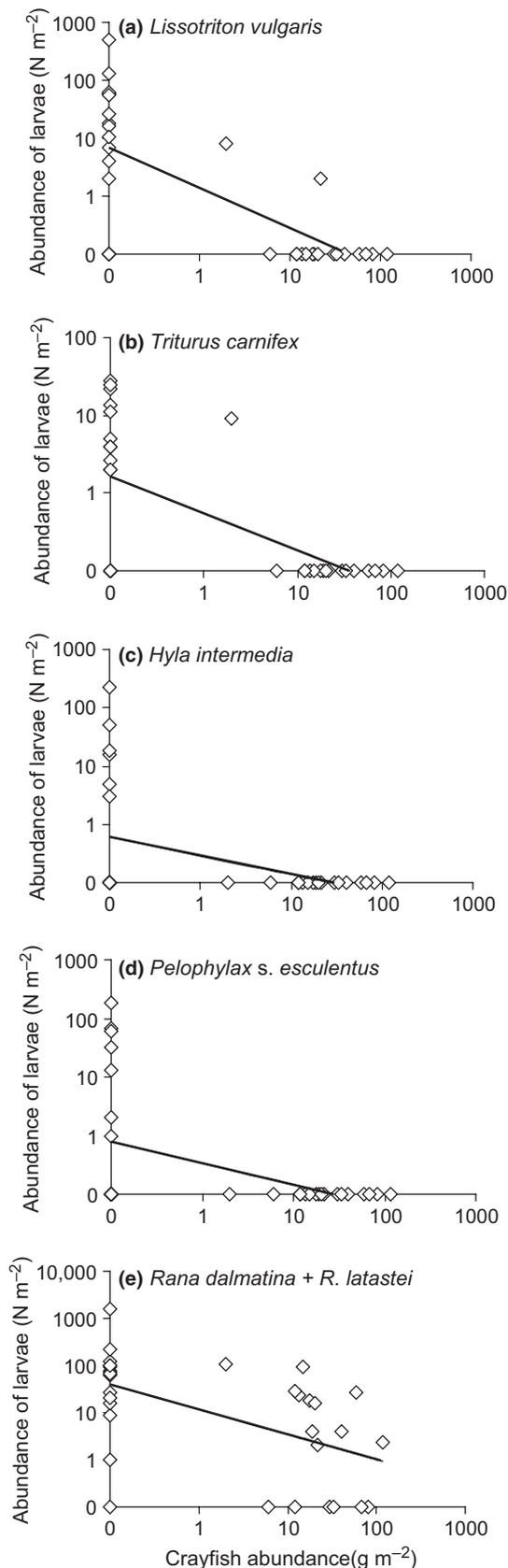


Figure 2 Relationship between the abundance of the invasive crayfish, *Procambarus clarkii*, and the abundance of amphibian larvae in 34 wetlands.

suitable breeding wetlands is already limited, and loss of breeding sites may quickly disrupt metapopulation dynamics and cause local extinction (Ficetola & De Bernardi, 2004).

Other species, such as brown frogs and pool frogs, often bred in invaded sites, still the frequency and abundance of larvae was very low there, suggesting that larvae are heavily predated. Experiments demonstrated that *P. clarkii* is able to prey on larvae of most European amphibians (Gherardi *et al.*, 2001; Cruz & Rebelo, 2005); crayfish predation is therefore the most likely cause of the negative relationship between crayfish and tadpole abundance (Fig. 2). The relationship was particularly clear for brown frogs. Brown frogs laid eggs in all ponds monitored through pipe sampling, but we detected very few or no tadpoles in the wetlands with high crayfish density (Fig. 2e). *Procambarus clarkii* invaded the study area only in the last years (five years before the beginning of this study, or less; Fea *et al.*, 2006). For several amphibians, lifespan is equal or even longer than this value (Lanza *et al.*, 2007); therefore, adults may continue to attempt breeding in invaded wetlands despite poor fitness. Some amphibians are able to detect the presence of predators in wetlands and avoid breeding in these environments (Resetarits, 2005). However, in Europe the presence of native crayfish that live in ponds and prey on amphibians is very limited (Souty-Grosset *et al.*, 2006); therefore, it is unlikely that European frogs evolved the ability to identify and avoid wetlands with crayfish. Explosive breeders, such as brown frogs and toads, can be particularly unable to detect the presence of predatory crayfish, because females stay in breeding wetlands only for a few hours.

Nevertheless, our measure of larval abundance cannot be used as a proxy of larval survival, because absence of larvae may indicate either low reproductive effort or high larval mortality. Furthermore, density-dependent effects are possible: at low densities, amphibian larvae can attain better survival and faster growth rate. Therefore, the heavy predation of larvae might not strongly decrease the number of metamorphs (Vonesh & De la Cruz, 2002). An accurate estimate of fitness would require an exhaustive measurement of the number of eggs laid, and the use of drift fences to capture all metamorphosing individuals. Such an estimate can be performed in one or a few small ponds (Karraker & Gibbs, 2009; Dodd, 2010), but is extremely difficult at large scale. On the other hand, measurement of larval abundance may be considered a good proxy of the overall reproductive success (see e.g., Werner *et al.*, 2009), because for several species (smooth newt, common toad and *Rana* frogs), larval sampling was performed immediately before the onset of metamorphosis. Indeed, in many invaded ponds, we detected amphibian breeding, but we did not detect larvae (Fig. 2e), suggesting that reproductive failure may be almost complete in some areas. Therefore, we believe that information obtained through larval sampling is useful to compare breeding performance among ponds. Finally, our study covered only a limited time span. Amphibian populations may undergo demographic fluctuation, and long-term studies are required for a clear understanding of the effects of IAS (Cruz *et al.*, 2008; Witte *et al.*, 2008).

Table 4 Models relating the abundance of amphibian larvae to environmental features and abundance of *Procambarus clarkii*: comparison of models with and without the effect of *P. clarkii*.

Species	SEVM	Environmental only				Environmental + <i>P. clarkii</i>				F	d.f.	P
		K	R ²	AIC	w	K	R ²	AIC	w			
<i>L. vulgaris</i>	Y	4	0.52	132.2	0.08	5	0.61	127.4	0.92	6.4	1,29	0.017
<i>Triturus carnifex</i>	N	1	–	123.9	< 0.01	2	0.36	110.6	0.99	24.6	1,32	0.0002
<i>Hyla intermedia</i>	N	3	0.03	132.3	0.16	4	0.17	129.0	0.84	5.0	1,30	0.032
Agile frogs	N	6	0.08	160.3	< 0.01	7	0.39	147.9	0.99	14.1	1,27	0.0008
<i>Pelophylax synklepton esculentus</i>	N	3	0.02	133.7	0.07	4	0.20	138.9	0.93	7.1	1,30	0.012

Significance values refer to the effect of *P. clarkii*; spatial eigenvector mapping (SEVM) was used for *Lissotriton vulgaris* only, because of the spatial autocorrelation of residuals. See Table 2 for abbreviations.

It has been proposed that native species may change habitat use in response to the invasion of alien predators, for example by shifting to suboptimal environments where IAS are absent (Creel *et al.*, 2005; D'Amore *et al.*, 2009). *Procambarus clarkii* is a generalist that can survive also in temporary wetlands, but it is more frequent in large, permanent waterbodies: a long hydro-period allows a prolonged activity period, while reproductive success can be limited in ephemeral wetlands (Gutiérrez-Yurrita & Montes, 1999; Cruz & Rebelo, 2007; Ficetola *et al.*, 2011b). This hypothesis would predict that amphibians shift their habitat preferences and select more temporary wetlands, where suitability for the crayfish is lower. However, we did not detect such a shift of habitat preferences. Species–habitat relationships (Tables 2 and 3) were similar to the ones detected by previous studies in uninvaded areas (e.g., Pavignano *et al.*, 1990; Ficetola & De Bernardi, 2004; Denoël & Ficetola, 2008; Indermaur *et al.*, 2010). In practice, most species were associated with relatively large and permanent wetlands, contrary to the predictions of the habitat use change hypothesis (Fig. 1). Shifts in habitat use may be caused by learning, or by natural selection running against individuals breeding in certain environments. For instance, amphibians can quickly evolve adaptations in response to environmental changes (including the introduction of hitherto absent species) that affect fitness in aquatic environments (Skelly & Freidenburg, 2000). Again, the short time since crayfish invasion probably prevented adaptations. Future studies assessing habitat shifts would be valuable to understand evolutionary or behavioural responses to IAS.

When a new IAS invades a biota, the consequences may not be evident for long periods (Strayer *et al.*, 2006). Early understanding of the impact over native species may allow more prompt and successful management actions. In our study case, considering the distribution of adult amphibians only would not reveal the negative effects of the crayfish and lead to incomplete conclusions. Presence/absence data are less sensitive to change, because the first negative effects of alien species occur on abundance. Adult amphibians may continue to attempt breeding in invaded wetlands, despite the very limited larval survival. Measuring the impact on fitness components, such as reproductive success, can be more complex and

resource consuming than collecting distribution data. Nevertheless, this approach allows a more clear understanding of the ongoing processes and of the consequences that IAS will have in the next future. This information may allow managers to start conservation actions before than consequences of IAS become irreversible. In this study case, management actions may include the control of crayfish abundance in the wetlands with the highest suitability for amphibians, through mechanical, physical, chemical or biological approaches, and the prevention of invasion in new areas (Gherardi & Panov, 2009).

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SUPPORTING INFORMATION

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

Figure S1 Results of constrained redundancy of species distribution, using species occupancy estimated using PRESENCE.

Table S1 Species distribution models using probability of presence, as estimated by PRESENCE occupancy models.

Table S2 Models relating the abundance of amphibian larvae to environmental features and abundance of *P. clarkii* (measured as individuals m⁻²).

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BIOSKETCHES

G. Francesco Ficetola is a postdoc working on ecology and conservation of amphibians, with a special interest on the issues of invasive species. This study is part of the PhD work of **Matteo E. Siesa** investigating issues associated with the invasion of *Procambarus clarkii*.

Author contributions: G.F.F., M.E.S., L.B., F.D. and E.P.S. designed the study; G.F.F., M.E.S. and R.M. performed fieldwork; G.F.F. performed analyses and wrote the first draft of the paper; all authors discussed the results and commented on earlier versions of the manuscript.

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