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Freshwater communities and biological invasions:

Odonata, Amphibia and *Procambarus clarkii*

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Cover illustration, from left to right: *Procambarus clarkii* adult male, *Lestes sponsa* adult male, and *Rana latastei* adult female.

Images by M. E. Siesa.

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ABSTRACT

This research work analyses processes and dynamics occurring during the early stages of a biological invasion in freshwater habitats.

I analysed processes determining the *Procambarus clarkii* invasion and the impact on native amphibians and odonates that have complex life cycles, I surveyed 148 among temporary and permanent wetlands of running and standing waters in a region that is at the edge of the invasion range of *P. clarkii*, I performed repeated sampling sessions in each wetland obtaining both qualitative and quantitative data on freshwater communities, I characterized each wetland using standard parameters, and I used GIS software for the description of the surrounding landscape. I analysed all data using spatial models and considering the spatial autocorrelation (for details see chapter 2).

Data analysis showed that the environmental features are important in determining the early phases of the crayfish invasion; in the study area, *P. clarkii* spreads more frequently in association with large and permanent wetlands in human-altered landscapes, and the autocorrelation of its populations is stronger at distances up to 2500 m suggesting that dispersion affect invasion processes up to this distance (chapter 2).

Further analysis showed that environment shapes both alien invasive species (AIS) and freshwater communities, but considering the optimal environmental features for *P. clarkii* and for amphibians and odonates, I observed important differences related to wetland size, depth, hydroperiod, exposition, presence of aquatic macrophytes, and surrounding landscape features (chapters 2, 3, and 5).

The observed relationships among distribution and composition of native communities and distribution of *P. clarkii* suggests that the invasive crayfish has only a limited direct impact on adult amphibians and odonates, but the analysis of juvenile stages of native communities (larvae of amphibians, larvae and exuviae of odonates) showed the dramatic loss of their abundance and richness in wetlands invaded by the

crayfish, indicating that, despite adults attempt reproduction in invaded sites, *P. clarkii* causes, with its activities, the fall down of their reproductive success (chapters 4 and 5).

This research confirms the strong negative impact of AIS on native communities. My data indicate that *P. clarkii* determines the loss of reproductive sites, and the decrement of the reproductive success when native populations use the invaded sites for breeding (chapters 4, 5, and 6).

This research highlights the importance of studying the early stages of an invasion, confirming that in this phase AIS populations have larger environmental needs that might make them more vulnerable at management actions (chapters 2, 3, and 6).

Through the analysis and description of the mechanisms determining the AIS impact on native communities, this research helps to identify the ongoing processes at early stages of the AIS invasion, and the consequences that the invasive crayfish will have in the near future, allowing managers to start conservation actions before that the invasion consequences become irreversible.

RIASSUNTO

Questa ricerca analizza i processi che si verificano durante gli stadi precoci di una invasione biologica negli habitat d'acqua dolce, in particolare, sono qui analizzati i meccanismi che determinano l'invasione di *Procambarus clarkii* e l'impatto di questo gambero sulle comunità autoctone degli anfibi e degli odonati, organismi che hanno cicli vitali complessi, con fasi sia acquatiche sia terrestri.

Sono stati studiati 148 differenti habitat acquatici, sia lotici sia lentici, con idroperiodo sia temporaneo sia permanente. Gli habitat acquatici indagati sono situati nell'Italia nord-occidentale, in un territorio che è stato solo recentemente invaso da *P. clarkii* e che costituisce il limite nord del suo areale italiano.

All'interno di ciascuna area umida sono state eseguite ripetute sessioni di campionamento che hanno consentito la raccolta di dati, sia qualitativi sia quantitativi, relativi alla composizione e distribuzione delle comunità studiate.

Ciascuna area umida è stata caratterizzata utilizzando un set di parametri standard mentre per la descrizione del paesaggio circostante è stata utilizzata la cartografia tematica della Regione Lombardia elaborata in ambiente GIS (per dettagli vedere il capitolo 2).

Tutti i dati raccolti sono stati analizzati utilizzando modelli statistici spaziali che considerassero l'autocorrelazione spaziale esistente tra le variabili analizzate.

L'analisi dei dati ha mostrato che le caratteristiche ambientali hanno una importanza significativa nel determinare le fasi precoci dell'invasione di *P. clarkii*; infatti, nell'area indagata, *P. clarkii* ha mostrato una significativa associazione con zone umide ampie, permanenti e circondate da un paesaggio ad elevata alterazione antropica. La distribuzione spaziale delle popolazioni di *P. clarkii* ha inoltre mostrato di risentire dell'effetto dell'autocorrelazione spaziale sino ad una distanza di 2500 m suggerendo che le dinamiche dell'invasione di *P. clarkii* siano influenzate dalla dispersione sino a questa distanza (capitolo 2).

Analisi successive hanno mostrato importanti differenze nei parametri che rendono l'ambiente ottimale per ciascuno dei taxa considerati (*P. clarkii*, anfibi e odonati). In particolare sono state osservate differenze relative a profondità, idroperiodo, estensione ed esposizione dell'area umida, presenza di macrofite acquatiche e caratteristiche del paesaggio circostante (capitoli 2, 3 e 5).

Le analisi effettuate sulla relazione tra la composizione e la distribuzione delle comunità autoctone degli anfibi e degli odonati e la distribuzione delle popolazioni di *P. clarkii* hanno suggerito l'esistenza di un limitato impatto diretto del gambero invasivo sugli stadi adulti degli anfibi e degli odonati, ma le analisi compiute sugli stadi giovanili delle comunità autoctone (larve degli anfibi, larve ed esuvie degli odonati) hanno mostrato la forte riduzione della loro abbondanza e ricchezza nelle zone umide invase dal gambero indicando che, nonostante i tentativi da parte degli individui adulti di riprodursi in tali siti, il gambero ne provoca la riduzione del successo riproduttivo (capitoli 4, 5 e 6).

I risultati ottenuti sottolineano l'importanza di studiare gli stadi precoci di una invasione, confermando che, in questa fase, le specie alloctone hanno richieste ambientali che possono accrescere il successo delle azioni di gestione (capitoli 2, 3 e 6).

Questa ricerca conferma il forte impatto negativo delle specie invasive sulle comunità autoctone: la presenza di *P. clarkii* determina la perdita di siti riproduttivi e la riduzione del successo riproduttivo quando le specie autoctone utilizzano i siti invasi per la riproduzione (capitoli 4, 5 e 6).

Attraverso l'analisi dei meccanismi che determinano l'impatto delle specie invasive sulle comunità autoctone, questa ricerca fornisce indicazioni utili ad identificare i processi in atto nelle prime fasi dell'invasione e le conseguenze che la diffusione del gambero invasivo avrà nel prossimo futuro. Queste conoscenze potrebbero guidare verso l'avvio di azioni di conservazione prima che gli effetti dell'invasione diventino irreversibili.

1.

Thesis structure and introduction

1.1. THESIS STRUCTURE

This thesis work analyses the relationships and dynamics that occur during early stages of a biological invasion. I considered freshwater communities and the invasion processes are here studied from multiple perspectives. I analysed both processes determining the invasiòn and effects on native populations.

I studied the red swamp crayfish, *Procambarus clarkii*, considered a keystone alien invasive species, and both amphibian and odonate communities, which are well known taxa already used as indicators for biological assessment and environmental monitoring (Córdoba-Aguilar, 2008; Linder et al., 2003; Wells, 2007).

I surveyed a total of 148 wetlands in Lombardy, Northern Italy, within the upper Po River plain, and the hilly pre-Alpine region called Brianza (Figure 1); this region is characterized by a sub-continental temperate climate, with annual average temperatures between 10° C, and 14.4° C, and annual average rainfall between 1400 mm and 1600 mm (Rogora et al., 2002). This region is rich in wetlands, forming a complex network of lakes, ponds, rivers, and canals of various size. I studied both standing and running waters including small lakes (Lake Varese, Lake Annone, Lake Pusiano, Lake Alserio, Lake Segrino, and Lake Montorfano), rivers and streams (River Ticino, River Lambro, Stream Curone, and Stream Nirone), and a wide variety of lentic and lotic biotopes like bogs, marshes, ponds, canals, springs, and fontanili. I investigated both temporary and permanent habitats located at an altitude between 132 m of Bernate Ticino and 397 m of Lake Montorfano.

Currently this region is at the edge of the invasion range of *P. clarkii* including areas with published and unpublished records (Barbaresi and Gherardi, 2000; Fea et al., 2006; Romanò and Riva, 2002), as well as areas where *P. clarkii* has not been recorded, but the species might be present because of proximity to localities of presence and existence of potentially suitable wetlands (Cruz and Rebelo, 2007; Gherardi, 2006).
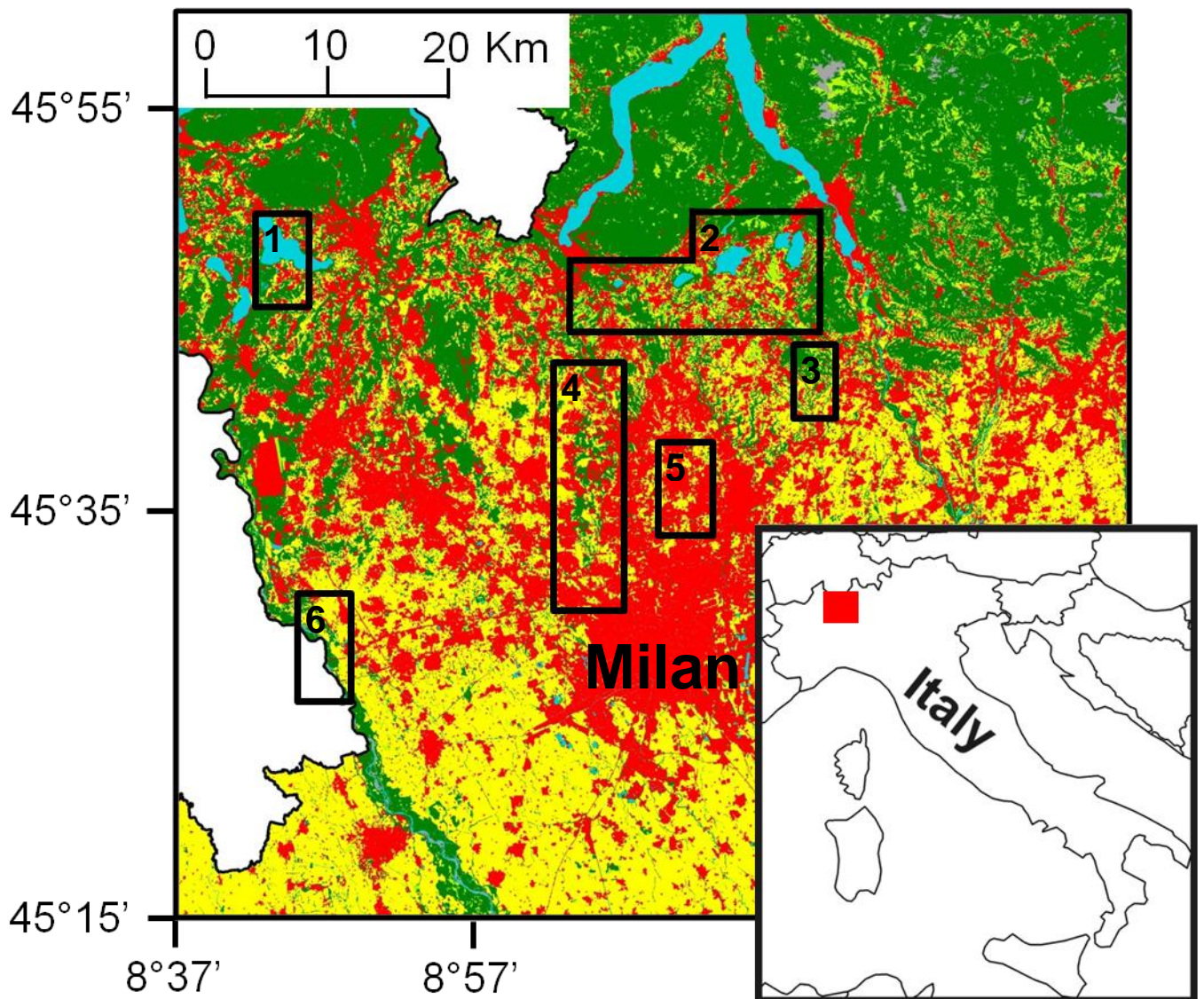
Figure 1. Study area; black lines enclose the areas where the surveyed wetlands are located; 1: Lake Varese and Regional Nature Reserve Brabbia Marsh; 2: River Lambro, Lake Annone, Lake Pusiano, Lake Alserio, Lake Segrino, Lake Montorfano, and Albate Marsh; 3: Montevecchia and Valle del Curone Regional Park; 4: Groane Regional Park; 5: Grugnotorto Park; 6: River Ticino. Map derived from the CORINE land cover; red: urban areas; yellow: cultivated areas; green: natural vegetation (mainly woodlands); grey: sterile areas; blue: water bodies.

Invasion dynamics are determined by multiple processes embedded within a complex spatial context (McIntire and Fajardo, 2009); invaded ecosystems show a complex web of interactions that involves environmental stressors, alien species and native communities (Didham et al., 2007; Strayer, 2010); therefore, the identification of ongoing mechanisms can be difficult, and only a small subset of studies considered the effects of alien invasive species (AIS) concurrently to the ones of environmental alterations, such as urbanization or the degradation of natural environments, that can have both negative and non-additive interactions with native species (Didham et al., 2007).

This thesis is composed by an introduction on the biology and ecology of the studied organisms, by four sections (chapters 2, 3, 4, and 5) that are four partially independent papers, in which the different lines of this research are discussed, and by a conclusion (chapter 6), where I discuss the main results and implications of this research work.

The first paper (chapter 2) analyses the *P. clarkii* spatial patterns and the mechanisms driving its invasion, to understand how environmental features influence the spread of this species. The problem is analysed using information theory, and spatial statistics (McIntire and Fajardo, 2009). Are tested four *a priori* hypotheses representing different biological processes that can explain the invasion of *P. clarkii*: (1) Connectivity; (2) Landscape alteration; (3) Wetland features; (4) Vegetation hypothesis. Understanding the mechanisms of an invasion allow to identify areas where the invasion risk is highest, helping to undertake more effective management and control actions (Hulme, 2006).

The second paper (chapter 3) shows the relationships between wetland features (hydroperiod; wetland size) and the distribution of amphibians and *P. clarkii*, to evaluate whether wetland features determine suitability in a different direction for these taxa. This analysis helps to focus management efforts within the areas where suitability for amphibians is high, while environmental features are suboptimal for *P. clarkii*.

The third paper (chapter 4) assesses the relationships between the distribution of amphibian breeding sites and *P. clarkii*, while taking into account environmental features;

subsequently, it analyses the abundance of amphibian larvae in a subset of wetlands where amphibians breed, testing whether *P. clarkii* causes a loss of breeding success (a key parameter of fitness). The distribution of both native and alien species can be strongly affected by spatial autocorrelation: nearby localities can have similar occupancy, because species distribution is limited by dispersal mechanisms (particularly in AIS), and because species biotopes are related to environmental features (e.g., vegetation, climate, and landscape composition) which are, in turn, spatially autocorrelated (Beale et al., 2010); therefore, spatial autocorrelation was integrated into all analyses.

The fourth paper (chapter 5) assesses the relationship between *P. clarkii* and the richness of the odonate communities. The distribution of adult odonates, larval stages, and exuviae, that indicate the odonate reproductive success (Córdoba-Aguilar, 2008), are analysed considering the landscape composition, the wetland features, and the spatial autocorrelation, to explain the observed community patterns at early stages of the *P. clarkii* invasion (Hamasaki et al., 2009), to suggest long term effects of this AIS on odonate communities, and to propose management lines.

The fallowing paragraphs (paragraphs 1.2, 1.3, 1.4 and 1.5) introduce the model species, to familiarize with their biology and ecology; amphibians and odonates are attractive organisms that have complex life cycles (Stoks et al., 2008; Wilbur, 1980) with adults and juvenile stages that occupy different niches and are subjected to different impact from environmental stressors. This work analyses this biological complexity allowing a detailed description of the ongoing phenomena and mechanisms driving alien species invasions and determining their impact on native communities.

1.2. ALIEN INVASIVE SPECIES (AIS)

With their usual functioning ecological systems produce services that are essential to preserve life on earth and that significantly contribute to human welfare. The biodiversity is an essential part of the ecological systems and biodiversity conservation is necessary to safeguard the ecosystem functions and life (Vilà et al., 2010; Worm et al., 2006).

In last decades, human activities have strongly contributed to the dramatic acceleration of biodiversity loss and now this process is clearly detected at global scale (Costanza et al., 1997; Daily et al., 1997; Drake, 2009; Worm et al., 2006). As the world's global economy increases scale, trends of transport, and travel activities, as dramatically enhances the spread of alien species, allowing them to easily surmount natural geographic barriers and accelerating the invasion processes (Wittenberg and Cock, 2001). As a result currently there are approximately 50.000 alien species in the United States (Pimentel et al., 2005), 11.000 alien species are inventoried in Europe (Drake, 2009), and more than 60% of alien insects and 25% of alien plants in Europe arrived during the last 50 years (Pyšek et al., 2010).

Alien species are organisms that are found out of their natural distribution area, and that could not be there without the human mediation. In some cases alien species can take advantage from the new environmental and biological features, or from human made habitat alterations (Bulleri and Airoldi, 2005; Didham et al., 2007; Schlaepfer et al., 2010); then, some alien species can quickly increase their abundance and distribution becoming invasive (AIS), and strongly affecting natural ecosystems by competing native species, consuming them, preying them, overgrowing them, spreading diseases, and hybridizing with them. Negative impact of AIS comprises alteration of hydrology, fire regimes, food web, flux of nutrients and other ecosystem processes, and can lead to the collapse of the invaded habitats. The AIS impact is now recognized as one of the major drivers of biodiversity loss both at local and at global scale (Pimentel, 2002); in fact, many native

ecosystems have been irretrievably invaded by AIS, leading to the extinction of thousands native species (Drake, 2009; Wittenberg and Cock, 2001).

The AIS introduction is often unintentional (Vitousek et al., 1997) but biological invasions should be considered a further impact of human activities on natural environments, determining enormous additional costs on agriculture, forestry, fisheries, and other human enterprises, as well as on human health. The financial costs of an invasion can be grouped by its detrimental effects on provisioning ecosystem services and by the costs needed to manage the AIS. Nevertheless, the economic evaluation of alien species cannot be based exclusively on market costs, and must include indirect and non-use value costs (Pejchar and Mooney, 2010; Vilà et al., 2010).

The evidence of increasing numbers of alien species introductions over the past few decades must contribute to the development of a global and regional awareness and to devote further researches to understand the AIS impact. The European DAISIE consortium ''Delivering Alien Invasive Species Inventories for Europe'', founded by the sixth framework program of the European Commission, has the aim to create an inventory of alien species that threaten European environment, providing the basis for spread prevention and control of them, and highlighting risks and impacts of invasions.

There are two main stages in the invasion processes: first, the introduced species must arrive, survive, and establish itself in a new area; second, the alien species, developing an invasive behavior, spreads and replaces native species; the number of individuals introduced and the number of release events have emerged as the most important factors for predicting whether or not an alien species will become established (Allendorf and Lundquist, 2003).

Alien species are not equally distributed among the different habitats of a region; in European habitats, can be recognized two major groups of AIS: plants and insects versus vertebrates (Pyšek et al., 2010). These two groups appear to be complementary in terms of habitat use: the highest number of alien plants and insects are found in human-made

urban or cultivated habitats. Conversely, vertebrate invaders are more distributed among cultivated land and habitats with aquatic and riparian woodlands.

Freshwater habitats are particularly vulnerable to alien species invasions: hundreds of freshwater species have been moved outside of their native ranges by a number of vectors such as canals, ballast waters, or by deliberate introductions, and releases from aquaria, gardens, and bait buckets (Strayer, 2010), and the alien species introductions into freshwaters are today regarded as a main driver of biodiversity loss (Gherardi, 2007).

A disconcerting example of the large presence of alien species in freshwater ecosystems is provided from Italian inland waters where 112 non native animals have been already recognized (64 invertebrates and 48 vertebrates) constituting the 2% of the total inland water fauna, and including seven AIS listed among the 100 worst invasive species at global scale (Gherardi et al., 2008); again, the rate of extinctions is impressive in freshwater habitats, even five times higher than those of terrestrial habitats (Ricciardi and Rasmussen, 1999) and AIS are a major cause of this biodiversity loss.

The most ecologically important AIS in freshwaters include pathogens that can destroy entire populations, aquatic plants that alter the quantity and quality of primary production and have strong engineering effects, molluscs that as primary consumers change the food web from its base, fishes that disrupt at various level the food web, and decapods acting as powerful omnivores and ecosystem engineers (Strayer, 2010).

1.3. THE RED SWAMP CRAYFISH *Procambarus clarkii*

Crayfishes are the largest mobile macroinvertebrates in freshwater ecosystems; they are an important component of freshwater habitats where their populations can strongly influence the environmental features (Holdich and Lowery, 1988).

Freshwater crayfishes occur naturally on all continents except Antarctica, the sub-continent of India, and Africa (they are present in Madagascar) (Souty-Grosset et al., 2006); North America and Australia are the two centers of highest crayfish diversity.

Taxonomically, crayfishes are Decapoda that belong to two superfamilies: Astacoidea (families Astacidae and Cambaridae) and Parastacoidea (family Parastacidae). Astacidae are distributed across Europe and in North America west of the Rocky Mountains while Cambaridae are distributed in North America east of Rocky Mountains, and south through Mexico and in Asia. Cambaridae is the largest freshwater crayfish family, with over 409 described species (Souty-Grosset et al., 2006).

Procambarus clarkii (Girard, 1852) is a cambarid crayfish native to north-eastern Mexico and south-central USA. For its dominant rule in culture and capture fisheries it is considered among the most important commercial species of crayfish; because of its economic and alimentary value it has been widely introduced and is now invasive worldwide with the exception of Australia and Antarctica (Barbaresi and Gherardi, 2000; Huner, 1994).

Within Europe *P. clarkii* was first introduced for aquaculture during 1973, in southern Spain, where soon

Figure 2. From top to bottom: *Procambarus clarkii* adult male, *P. clarkii* soon after moulting, and *P. clarkii* female with offspring attached to the abdomen.
Images by M. E. Siesa.

becomes established; afterwards, new introductions have followed throughout Europe, where is currently present in 13 countries, appearing for the first time in Italy during 1977 (Gherardi, 2006; Souty-Grosset et al., 2006).

The enhanced plasticity of *P. clarkii* life cycle (Gherardi et al., 2000) enable it to tolerate a wide range of physical, chemical, and biological conditions, it is a generalist crayfish that can live in a wide range of different wetlands, including rice fields, marshes, ponds, lakes, reservoirs, irrigation canals and flowing waters, from 0 to 1200 m a.s.l. that is its altitudinal limit in Europe (Souty-Grosset et al., 2006). *P. clarkii* can survive both in waterbodies that freeze during winter and in seasonally flooded waterbodies that, in its natural range, host the most abundant populations (Huner, 1988).

During the dry season *P. clarkii* makes burrows with a depth of more than 2 m that provide humid survival shelters (Correia and Ferreira, 1995; Cruz and Rebelo, 2007; Ilhéu et al., 2003). *P. clarkii* can survive for long period overland and shows a dispersal ability that can exceed 3 km per day (Barbaresi and Gherardi, 2000; Barbaresi et al., 2004; Gherardi et al., 2002).

Procambarus clarkii is considered an r-selected species with high fecundity, fast maturation and short life cycle, it can reach the maturity after eleven moults, with an intermoult period of 6–30 days at 20-22° C making possible two generations per year. Mating occurs during autumn winter and spring, when, depending on female size, *P. clarkii* can produce more than 700 eggs each oviposition, with embryonic development that lasts 2-3 weeks at 22° C. In addition, *P. clarkii* shows complex mother-offspring relationships that can enhance offspring survival, increasing the demographic growth of invasive populations (Aquiloni and Gherardi, 2008; Gherardi, 2006; Hazlett et al., 2003; Huner, 1988; Huner and Lindqvist, 1991; Savini and Occhipinti-Ambrogi, 2008; Souty-Grosset et al., 2006).

Procambarus clarkii is a keystone AIS that can have multiple negative impact on native species, ecosystems, and human health. *P. clarkii* can diffuse the crayfish plague *Aphanomyces astaci* and outcompete native European crayfishes, causing their striking

decline (Gherardi and Panov, 2009; Gil-Sánchez and Alba-Tercedor, 2006; Souty-Grosset et al., 2006); *P. clarkii* is a polytrophic and opportunistic crayfish that feeds on vegetal detritus but also on molluscs, insects, amphibians, fishes and macrophytes, causing complex changes in the food webs and leading to the decline of several native species (Cruz and Rebelo, 2005; Cruz et al., 2008; Ficetola et al., 2010; Gherardi, 2006; Siesa et al., 2010a; Siesa et al., 2010b; Souty-Grosset et al., 2006). *Procambarus clarkii* accumulates in its tissues heavy metals, pesticides and toxins of cyanobacteria, transferring them to its consumers (Alcorlo et al., 2006; Gherardi, 2006), it can also spread parasite helmints of the genus *Paragonimus*, potentially pathogenic for humans (Gherardi and Panov, 2009).

Moreover, *P. clarkii* can destroy submerged macrophytes using chelipads in non consumptive actions, it can damage agricultural areas such as rice plantations by feeding on young rice plants, and with its burrowing activity it can destabilize dams and banks, and cause release of nutrients from the sediment to the water (Barbaresi and Gherardi, 2000; Gherardi and Panov, 2009; Matsuzaki et al., 2009; Souty-Grosset et al., 2006).

Procambarus clarkii, that is considered one of the 100 worst alien species in Europe (Drake, 2009), acts as an ecosystem engineer which modifies the flow of nutrients and alters multiple features of invaded wetlands. In mesotrophic and eutrophic wetlands it can lead to a switch from a clear to a turbid water phase, with abundant microalgae and reduced primary production by hydrophytes and periphyton (Gherardi and Acquistapace, 2007; Matsuzaki et al., 2009). With its activities *P. clarkii* leads to a dramatic loss of the biodiversity and increase of biotic homogenisation (Correia and Anastacio, 2008; Cruz et al., 2008; Gherardi and Acquistapace, 2007).

Worldwide, main predators of *P. clarkii* are fishes and large wading birds like herons, egrets and storks; other predators are carnivorous mammals such as otters, minks, raccoons, turtles, snakes and alligators; invertebrates like dytiscid beetles, aquatic hemipterans, fisher spiders, and dragonfly larvae can also be effective predators of early juvenile stages of the crayfish (Correia, 2001; Souty-Grosset et al., 2006; Witzig et al., 1986).

The management of large invasive populations of *P. clarkii* is a challenge that led to multiples practical attempts and researches (Aquiloni et al., 2009; Aquiloni et al., 2010; Gherardi and Panov, 2009). In Spain organophosphate pesticides used by farmers caused the deaths of thousands of birds (Holdich and Lowery, 1988); in Italy the synthetic pyrethroid "ciflutrin" has been tested in laboratory and found to be relatively effective against the invasive crayfish (Souty-Grosset et al., 2006); biodegradables surfactants, which inhibit oxygen consumption, might have some potential in limiting the damage being done by *P. clarkii* in rice crops (Souty-Grosset et al., 2006); the sterilization by X-ray irradiation of a large number of males, then released in invaded habitats, is under research for its potential in reduction of the overall reproductive success of *P. clarkii* populations (Aquiloni et al., 2009); the potential of European eel (*Anguilla anguilla*), an effective predatory fish, was also tested to employ it in the biological control of *P. clarkii* (Aquiloni et al., 2010).

None of the so far attempted actions had a decisive effect in controlling invasive populations of *P. clarkii* (Aquiloni et al., 2009; Aquiloni et al., 2010). More effective management programs give primary importance to the prevention and early detection of new introductions, and adopt a combination of methods, that have to be calibrated with the application context (Aquiloni et al., 2005; Hein et al., 2007; Keller et al., 2008; Polasky, 2010).

1.4. ECOLOGY AND CONSERVATION OF AMPHIBIANS

For the complexity of their life cycles (Wilbur, 1980) and the degree to which their life cycles vary in response to environmental conditions, amphibians are exceptional among tetrapods.

In the "standard" life cycle of most amphibians, reproductive females lay eggs in water, larvae hatch, feed, and gradually metamorphose into terrestrial or semi-aquatic adults; larvae and adults of urodeles are carnivorous, while anuran larvae are mostly herbivorous and adults are carnivorous (Wells, 2007).

Amphibian larvae can modify their rates of development in response to environmental conditions such as temperature, hydroperiod, water depth, dissolved oxygen concentration, pH, competitors, larval density, food availability, and presence of predators. These factors, either independently or in concert, could affect larval growth and development, depending on the relative costs and benefits of remaining in the larval stage or, under unfavorable environmental conditions, to accelerate the metamorphosis (Wells, 2007).

Amphibian larvae affect primary production and nutrient cycle in aquatic biotopes: in pond ecosystems, the increased tadpole biomass observed during the life cycle of anuran populations can be accompanied by reduced standing crop of suspended particles, including phytoplankton, by a shift in the state of nitrogen from largely particulate to largely dissolved, and by reduced rates of primary production, with a shift in phytoplankton community

Figure 3. From top to bottom: *Rana latastei* adult female, *Hyla intermedia* first year juvenile, and *Salamandra salamandra* pregnant female.
Images by M. E. Siesa.

structure determining the reduction of filamentous algae; tadpoles can regulate the primary production by both reducing standing crop and altering specific growth rates of algae; when metamorphosis removes these transient consumers, rates of primary production can dramatically increase (Seale, 1980).

At all life stages amphibians constitute a food resource for a rich predator community; vertebrate predators include fishes, reptiles like turtles and snakes, birds, mammals, and even larval and adults urodeles and anurans; invertebrate predators worldwide are leeches, arthropods like amblypygids, spiders, aquatic bugs, and predaceous aquatic beetles. Larval odonates and crustacean decapods like crayfishes are further important predators of amphibians (Bowerman et al., 2010; Wells, 2007).

The amphibian population demography is complex to study because the life cycles of most amphibians include different stages being exposed to different predators, and to different physical and biological environmental features, an then, to understand what factors regulate the size of amphibian populations, studies are needed on complex processes occurring at all life stages (Hellriegel, 2000).

Studies analysing prey-predator interactions in amphibian populations, although include lists of potential predators, seldom provide quantitative data on the impact of those predators on amphibian survivorship, and the impact of specific predators is even more difficult to estimate; therefore data on the demographic impact of predation are currently scarce, at present, we need more information on sources of mortality at all stages of the life cycle for any amphibian species (Wells, 2007).

Habitat fragmentation and alteration is one of the major factors leading to the observed decline of several amphibian species (García-Muñoz et al., 2010). Amphibians are strongly sensitive to changes of environmental parameters, in North America and Europe, where there is an impressive and well documented decline of amphibian populations, this trend is usually associated with habitat alterations.

Because of their complex life cycle that force amphibians to a life between water and ground, amphibian populations survival is strongly dependent by the features of both

aquatic and terrestrial habitats; suitable terrestrial habitats surrounding breeding ponds are needed for amphibian persistence (Hazell et al., 2001). Wetlands surrounded by forests or scrublands or in watersheds less exploited by humans have an increased suitability for amphibians. The highest amphibian richness values have been found in wetlands where the watersheds are more heterogeneous, with numerous shelters and migration corridors which are essential for the survival during the terrestrial phase. Others important factors that can determine amphibian decline include the direct exploitation, the impact of pathogens, the global climate change (Stuart et al., 2008), the increased ultraviolet B (UV-B) radiation and the augmented predation pressure due to the introduction of invasive predators, like fishes or crayfishes, in aquatic biotopes where amphibians evolved in absence of them (Alford and Richards, 1999; Cruz et al., 2008); alien predators, such as the North American bullfrog *Rana catesbeiana* and the freshwater crayfish *P. clarkii*, can determine the collapse and the extinction of amphibian populations (Cruz et al., 2006b; Cruz et al., 2008; Siesa et al., 2009b).

Amphibians are considered the most threatened taxon between vertebrates, at present, the 43.2% of the amphibian species have declining populations and, applying the IUCN Red List criteria (IUCN, 2001), 32.5% of them (1856 species) are considered Vulnerable, Endangered or Critically Endangered, and a generalized increase of this percentage is expected in the next future. There is an urgent need of new data on the causes of amphibian decline, in fact, for the 22.5% of the amphibian species there is a remarkable lack of data on population demography, ecological needs, threats, and declining causes (Chanson et al., 2008; Stuart et al., 2008).

1.5. ECOLOGY AND CONSERVATION OF ODONATES

Odonates have a complex life cycle (Corbet, 2004; Wilbur, 1980) with incomplete metamorphosis; odonate life cycle includes egg, larva, and adult stage but no pupa, and comprises an abrupt ontogenetic change in individual's morphology and habitat (Stoks et al., 2008; Wilbur, 1980).

Eggs are laid either freely in water, inserted in the vegetation growing in the water, in the vegetal detritus, in the sediment, beside water, or in damp ground; after laying, eggs may enter diapause, to pass through harsh environmental conditions, or develop immediately, hatching from two, like most Zygoptera, to four weeks after laying. After hatching, individuals emerge as aquatic or semi-aquatic larvae and can remain as larvae for weeks, if they occupy temporary ponds, to years, in case of semivoltine species developing in permanent waters. At the end of the larval phase, individuals metamorphose into aerial adults that may survive for a few days, in the most of the species, to months, like the *Sympecma* genera that overwinter at the adult stage (Askew, 2004; Corbet, 2004).

Odonates are top predators in vertebrate free habitats (Simaika and Samways, 2008); both larvae and adults are carnivores, they are generalist feeders preying anything of suitable size such as leeches, molluscs, worms, crustaceans, midges, mosquito larvae, fish fry, and tadpoles (Askew, 2004; McPeek, 2008; Simaika and Samways, 2008). Larvae catch the prey detecting them with the antennae and with the eyes, and using a

Figure 4. From top to bottom: *Lestes sponsa* mature male, *Enallagma cyathigerum* mature male, and *Sympetrum sanguineum* immature female.
Images by M. E. Siesa.

specialized mouth part that differentiate from the labium and is called mask. The mask, a unique odonate adaptation, is a prehensile structure that can be shot out to grab the prey (Askew, 2004; Corbet, 2004; McPeek, 2008).

Although predators at all life stages odonates can play a wide set of functions in the food web of freshwater ecosystems that depend on the species, life stage, presence of other predators, and composition of the biological community in the biotopes (Corbet, 2004; Crumrine, 2010).

Several species of odonates are stenotopic and require specific habitat conditions (Oertli, 2008), and one of the primary environmental features that shape odonate communities is the habitat difference between flowing and standing waters; predators may also play an important role in determining the distribution of species; other important factors for odonate species distribution are the water quality, the sediment and the microhabitat substrate, the presence and type of the vegetation, the quantity and quality of the shadow, the extent of aquatic habitats, and the water depth (Corbet, 2004; Córdoba-Aguilar, 2008).

Odonates have a peculiar life cycle that put them in contact with the aquatic environment, during the juvenile phases, and with the surrounding landscape, from the emerging phase, giving them a particular sensitivity to human disturbance both at microhabitat and at landscape level (Samways and Steytler, 1996). Based on adult stage observation, it was evaluated the relative importance of the different habitat elements on odonate community composition, finding that the abundance of benthos preys and the within-habitat environment and landscape features have comparable effects in determining the adult odonate assemblages (Hamasaki et al., 2009; Yamanaka et al., 2009); in addition, odonate species richness appears to be associated with species richness of vascular plants both in the aquatic biotope and in the surrounding landscape (Hamasaki et al., 2009; Mabry and Dettman, 2010; Sahlen and Ekestubbe, 2001).

Odonate community composition and demography depend on the demographic rates of each life stage that are influenced by a wide set of physical, chemical, and biological parameters (Corbet, 2004; Pierce et al., 1985; Worthen et al., 2001).

After deposition, eggs may die or fail to develop because they are unfertilized, or the development may be arrested by unfavorable environmental parameters, furthermore, eggs can be parasitized or eaten (Fursov and Kostyukov, 1987).

Because many species spend the majority of their life as larvae, the larval stage is a demographically critical phase of the life cycle for determining both odonates distribution and abundance (McPeek, 2008). Although the demographic effects of parasites have been better studied in the adult stage, parasites are a possibly significant source of larval mortality and difficulty to growth. Some of the key parasites that infect odonate larvae are nematodes and microsporidians (McPeek, 2008).

Odonate larvae are frequently food-limited due to lower productivity habitats, or because of competition for resources with other organisms in the food web. In presence of mortality threats like conspecifics cannibals or other predators, odonate larvae reduce growth rates until more than 50%; reduced growth rates may be critical for species living in water bodies that dry periodically where fast juvenile phase is crucial; processes that slow growing, forcing odonate larvae to remain longer time in smaller size classes, increase larval exposure to potential mortality sources like cannibals and predators thereby increasing the larval mortality rate. Larval mortality due to predation is the major demographic force shaping abundances for most species (Crumrine, 2010; McPeek, 2008); habitually, the most important larval predators are fishes, other odonates (Crumrine, 2010), and other aquatic insects like predaceous diving beetles and aquatic hemipterans (Heads, 1985; Magnusson and Williams, 2006); experimental results reveal that up to 80% of larval mortality is due to the dominant predator present in the species biotope (McPeek, 2008; Stoks and McPeek, 2003).

Crayfishes, with their feeding activity, can drive to the decline of abundance and richness of macrophytes and macrobenthic invertebrates, dramatically altering the

odonate habitat and reducing richness and abundance of odonate communities (Gherardi and Acquistapace, 2007; Matsuzaki et al., 2009; McCarthy et al., 2006; Nyström et al., 1996; Ott, 2009; Ott and Samways, 2010; Siesa et al., 2009c; Wilson et al., 2004); bigger dragonfly larvae (e.g. Aeshnidae) are effective predators of young crayfishes (until 30 mm total length) (Gydemo et al., 1990; Witzig et al., 1986) but larger crayfishes can easily feed on odonate larvae, even preferring them to other type of prey (Ilhéu and Bernardo, 1993).

Odonates are the first insect order evaluated at global scale applying the IUCN Red List Categories and Criteria (IUCN, 2001); 10% of odonates are considered threatened and assigned to an IUCN Red List category among Vulnerable, Endangered, and Critically Endangered (Clausnitzer et al., 2009).

Odonates constitute an effective tool for environmental assessment and monitoring; when sampled using standardized methods, odonates provide excellent data for ecological and evolutionary studies, and are proposed as biodiversity indicators, for the assessment of water-body health, for the monitoring of management practices, for the detection and prediction of the biological impact of the climate warming, and for the evaluation of AIS impact (Oertli, 2008; Ott, 2007; Sahlen and Ekestubbe, 2001; Samways, 2008; Settele et al., 2010). Odonates are considered a keystone, umbrella, and flagship taxon; they are present in different aquatic biotopes and wetlands like marshes, peat bogs, ponds, lakes, and running waters; they are well diverse, well identifiable, and have a well established taxonomic framework; they use aquatic habitats as larvae and terrestrial areas as adults; they are geographically widespread, they are particularly well accessible and amenable to standard sampling; they are likely to generate political and popular sympathy; they are well studied, for many species are available data on ecological traits, and they have a significant conservation value. These reasons allow to recognize odonates as an interesting and helpful model to study the ecological interactions in freshwater ecosystems (Clausnitzer et al., 2009; Oertli, 2008; Sahlen and Ekestubbe, 2001).

2.

Spatial patterns and invasion processes of the crayfish *Procambarus clarkii*

ABSTRACT

The red swamp crayfish *Procambarus clarkii* is native to Eastern North America, but has been introduced worldwide. It is a keystone invasive species causing multiple issues to native ecosystems. Analysing spatial dynamics and distribution patterns can help to understand the mechanisms driving invasions. We assessed the distribution of *P. clarkii* in 119 waterbodies in a recently invaded area, through multiple techniques. We evaluated four *a priori* hypotheses on processes that may determine crayfish invasion: landscape alteration, connectivity, wetland suitability for abiotic and biotic features. We used spatially explicit statistical techniques (Spatial Eigenvector Mapping and Generalized Additive Models) within an information-theoretic framework to assess the support of hypotheses; we also analysed the pattern of spatial autocorrelation. The analysis of detectability showed high reliability of survey results. *Procambarus clarkii* was significantly associated to the largest, permanent wetlands within the most human-dominated landscapes, indicating that abiotic features of wetlands and landscape alteration are major drivers of the species' distribution. Species distribution data, residuals of ordinary regression models, and spatial eigenvectors all showed positive and significant spatial autocorrelation at distances up to 2500 m; this may be caused by the dispersal ability of the species. At the early stages of the invasion, this crayfish can take advantage of human activities; wetlands with high suitability may act as sources for the invasion. Our analyses help to understand the processes determining the invasion and to identify areas most at risk where screening and early management efforts can be focused.

KEYWORDS: a priori inference, habitat selection, isolation, landscape composition, spatial autocorrelation.

2.1. INTRODUCTION

The red swamp crayfish *Procambarus clarkii* is native of Eastern North America, but has been introduced worldwide because of its economic and alimentary value, as it is considered among the most important commercial species of crayfish, and is now invasive in five continents (Barbaresi and Gherardi, 2000; Huner, 1994).

Multiple features determine the high invasiveness of *P. clarkii*: it has great ecological plasticity, tolerating a wide range of physical, chemical, and biological conditions. Furthermore, *P. clarkii* is traditionally defined as an r-selected species that can produce, depending on female size, more than 700 eggs; complex mother-offspring relationships can enhance offspring survival increasing the demographic growth of invasive populations (Aquiloni and Gherardi, 2008; Gherardi, 2006; Hazlett et al., 2003; Huner and Lindqvist, 1991; Savini and Occhipinti-Ambrogi, 2008).

Procambarus clarkii is a keystone alien invasive species that can have multiple negative consequences on native species, ecosystems and human health. It is a polytrophic and opportunistic crayfish, feeding on vegetal detritus but also on molluscs, insects, amphibians, fishes and macrophytes, and therefore causing complex changes in food webs and leading to the decline in the abundance and diversity of several native species (Cruz and Rebelo, 2005; Cruz et al., 2008; Gherardi, 2006; Souty-Grosset et al., 2006). Furthermore, *P. clarkii* can diffuse the crayfish plague *Aphanomyces astaci* and outcompete native European crayfishes, thereby causing their decline (Gherardi and Panov, 2006; Gil-Sánchez and Alba-Tercedor, 2006; Souty-Grosset et al., 2006). *Procambarus clarkii* is also able to accumulate heavy metals, pesticides and toxins of cyanobacteria transferring them to its consumers(Alcorlo et al., 2006; Gherardi, 2006), and can spread parasite helminths of the genus *Paragonimus*, potentially pathogenic for humans (Gherardi and Panov, 2006). Moreover, *P. clarkii* can damage agricultural areas such as rice plantations, by feeding on young rice plants, and dams, canals, river and lakes where with its burrowing activity may destabilize banks (Barbaresi and Gherardi, 2000; Gherardi and Panov, 2006; Souty-

Grosset et al., 2006). Its excavation activities also cause sediment resuspension and nutrient release from the sediment to the water. Submerged macrophytes are destroyed using chelipads in non-consumptive actions. Therefore, *P. clarkiis* acts as an ecosystem engineer which modifies the flow of nutrients and alters multiple features of invaded wetlands (Matsuzaki et al., 2009).

The eradication or control of large, invasive populations of *P. clarkii* is challenging (Aquiloni et al., 2009; Aquiloni et al., 2010). Understanding the mechanisms determining the invasion dynamics can allow us to identify areas where the risk of establishment and invasion is highest, helping to tackle the invasion at the earliest stages and therefore increasing the effectiveness of management (Hulme, 2006). However, invasion dynamics are determined by multiple processes embedded within a complex spatial context (Gallien et al., 2010; McIntire and Fajardo, 2009); therefore, the identification of ongoing mechanisms can be difficult. Information theory, and recent development of spatial statistics constitute a robust framework to develop and test explicit hypotheses on the mechanisms determining spatial patterns of invasive species, and can therefore help to identify the mechanisms driving species invasion (McIntire and Fajardo, 2009).

This study analysed the spatial dynamics and the diffusion pattern of *P. clarkii*, to understand how environmental features influence the spread of this species. We considered four *a priori* hypotheses representing different biological processes that can explain the invasion of *P. clarkii* (Table 1). (1) Connectivity hypothesis: large, permanent wetlands or rivers can act as sources of invasive populations, therefore invasion risk is highest close to these large waterbodies (Cruz and Rebelo, 2007; Rahel, 2007). (2) Landscape alteration hypothesis: increased environmental disturbance due to human activities favours synanthropic species including many invasive species; furthermore introductions can be more frequent in human dominated areas. Therefore, human alteration of landscape can increase the risk of introduction and invasion (Cutway and Ehrenfeld, 2009; Ficetola et al., 2007; King and Tschinkel, 2008; Leprieur et al., 2008). (3) Wetland features hypothesis: *P. clarkii* is often associated to large, permanent wetlands,

therefore hydrological and morphological features of waterbodies can determine the likelihood of establishment and invasion (Cruz and Rebelo, 2007). (4) Vegetation hypothesis: vegetation within and nearby wetlands is a key feature of waterbodies that can have strong effects on animal communities (Van Buskirk, 2005), and therefore can be important also for the establishment of *P. clarkii*. We analysed an area of Northern Italy where *P. clarkii* has been introduced only recently; the study area is at the leading edge of the invasion range (Barbaresi and Gherardi, 2000; Fea et al., 2006). In this area, the abundance of *P. clarkii* is currently limited, therefore our analysis would allow to identify the mechanisms most important at the early stages of the invasion, i.e., the stages at which management and control can be more effective (Hulme, 2006).

2.2. METHODS

Study area and surveys

We considered 119 waterbodies (ponds, slow stream ditches and small lakes) in Lombardy, Northern Italy, within the upper plain of the Po river at altitudes of 132-397 m a.s.l. (Figure 1). Annual average temperature is 10°-14.4° C and annual mean rainfall is 1400-1600 mm (Gerletti and Marchetti, 1977). This region is rich in wetlands, with a complex network of lakes, ponds, rivers and canals of varying size. The study area is currently at the edge of the invasion range of *P. clarkii*; we surveyed areas with published and unpublished records of *P. clarkii* (Barbaresi and Gherardi, 2000; Fea et al., 2006; Romanò and Riva, 2002), as well as areas where *P. clarkii* has not been recorded, but the species might be present because of proximity to localities of presence and the existence of potentially suitable wetlands.

We surveyed each wetland seven times from March to August to assess the distribution of *P. clarkii*. We used multiple techniques to evaluate species presence, including nocturnal visual encounter surveys, repeated dip-netting of the wetland banks,

bottom and vegetation, the identification of burrows and exuviae (Reynolds et al., 2006). Three surveys were held after sunset, using multiple light sources to perform night-viewing and 4 surveys were performed during daylight. At each survey, multiple researchers sampled each site for about 20 min. We recorded relative humidity and air temperature at each survey; data were recorded in the field using pre-printed tables and a personal digital assistant equipped with GPS and ArcPad GIS software.
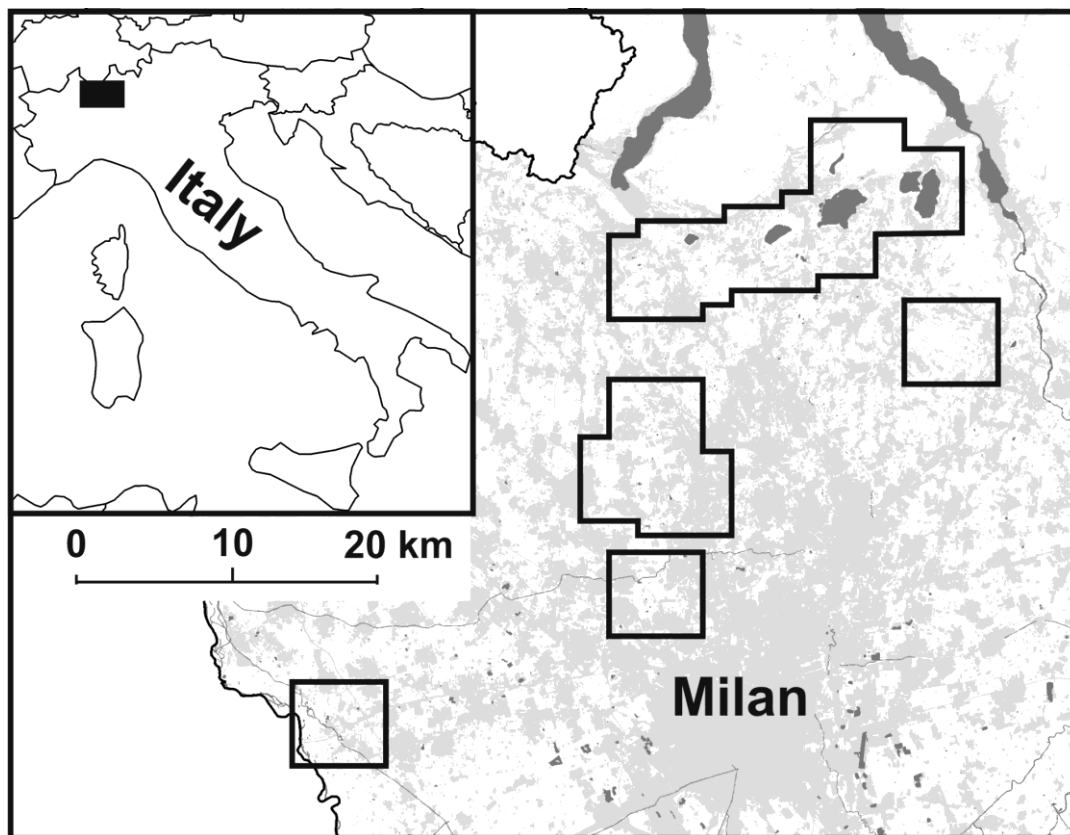

Figure 1. Study area (Lombardy region, Northern Italy). Black lines enclose the areas where wetlands are located, dark shaded areas represent lakes and major rivers, light shaded areas represent urban areas.

Environmental variables

We measured 10 environmental variables describing features of wetlands and the surrounding landscape, and representing the four a priori hypotheses on factors determining the diffusion of *P. clarkii* (Table 1). *Connectivity hypothesis*. We measured the overland distance to the nearest river, lake or large pond (surface ≥ 1ha). We used overland distance because previous studies showed that this measure may be more appropriate than distance along the hydrographic network, particularly in still water (Cruz and Rebelo, 2007). *Landscape alteration hypothesis*. We measured urban cover % in a radius of 400 m from the wetland. We used a 400 m radius as previous studies showed landscape features at this scale influence the distribution of other species living in similar wetlands (Ficetola et al., 2009); preliminary analyses performed at different scales (100 m; 1000 m) yielded very similar results. We measured landscape features (connectivity, urban cover) on the basis of the regional Vector Map of the Lombardy region (2008 land use maps; www.cartografia.regione.lombardia.it). *Wetland features hypothesis*. For each wetland we recorded three abiotic features: Maximum depth, surface area, and hydroperiod (0: temporary, i.e. dry during at least one survey; 1: retained water during all the samplings) (Table 1). *Vegetation hypothesis*. In May, for each wetland we measured four features describing the vegetation of the wetland and of the nearby areas: percentage of canopy cover, percentage of shoreline and emergent vegetation of the wetland, cover of grass and shrubs within 30 m from the wetland. The measurement of vegetation variables was performed following standard assessment protocols for details see Table 1 (Ficetola and De Bernardi, 2004; Knutson et al., 2004; Werner and Glenmeier, 1999).

Statistical analyses

A site is determined to be occupied by a given species if it is detected at that site, but non detecting a species during all sampling occasions does not necessarily indicate the species is absent. We therefore used PRESENCE (Hines, 2006; MacKenzie et al., 2006)to evaluate the probability of occupancy of sites where we did not detect it. For the analysis, we assumed that probability of detection can be affected by Julian date, hour of survey, air temperature, and humidity %.

Some of our a priori hypotheses were represented by multiple environmental variables (Table 1). This can pose problems for the analyses, because variables representing the same process are inherently correlated, and intercorrelated variables can bias regression estimates (Berry and Feldman, 1985). Furthermore, the number of variables included in an analysis affects its power and the Akaike's Information Criterion (see below). For this reason, we used principal component analysis (PCA) to reduce the variables representing the same hypothesis to a lower number of uncorrelated components. For the variables describing wetland features, PCA extracted a single component representing 49% of variation of original variables; this variable (hereafter: WETLAND) was positively correlated to wetland area (r = 0.56, P < 0.001), depth (r = 0.83, P < 0.001) and permanent hydroperiod (r = 0.68, P < 0.001). For the variables describing vegetation features, PCA extracted two components. The first component (VEGETATION_1) explained 43% of variance, and was significantly correlated to riparian vegetation (r = 0.88, P < 0.001), surrounding grass (r = 0.90, P < 0.001) and shrubs (r = 0.74, P < 0.001); the second component (VEGETATION_2) explained 26% of variance, and was significantly correlated to canopy cover (r = 0.81, P < 0.001), riparian vegetation (r = -0.21, P = 0.02), emergent vegetation (r = 0.74, P < 0.001) and surrounding shrubs (r = 0.24, P = 0.009). In our analyses, we used the variables extracted by PCA instead of the original variables.

Several methods have been proposed for regression analysis of spatial data and performance can be different among the various approaches, thus we built our models using two different techniques (Beale et al., 2010; Bini et al., 2009; Dormann, 2007). First, we analysed data using spatial eigenvector mapping (SEVM). This method is relatively new, but is increasingly used for the analysis of ecological data. SEVM allows the translation of the spatial arrangement of data points into explanatory variables (eigenvectors) capturing spatial effects (Dormann, 2007). There are multiple implementations of SEVM, depending on how the eigenvectors to be included in the models are selected (Bini et al., 2009). In our implementation, we selected the eigenvector(s) best reducing the spatial autocorrelation of residuals, and then included as spatial predictors into generalized linear models (Dormann, 2007; Griffith and Peres-Neto, 2006). Comparisons among statistical methods showed that this method is flexible and efficient, also when analysing non-normal data (Bini et al., 2009; Dormann et al., 2007; Peres-Neto and Legendre, 2010). Furthermore, the identity of eigenvectors included into models has been proposed to be indicative of the scale at which autocorrelation takes effect (Diniz-Filho and Bini, 2005).

Second, we used generalized additive models (GAM), as implemented by Beale et al. (2010). In this implementation, we incorporated the coordinates of data in GAMs as covariates; we assumed linear relationships between species distribution and the environmental predictors representing our hypotheses (Beale et al., 2010). Simulations showed that GAMs are among the techniques with lowest bias and with good performance even in presence of violations of assumptions, such as non-stationariety of autocorrelation (Beale et al., 2010).

We used an information-theoretic approach, following the procedure detailed in Denoël et al. (2009), to evaluate the support of the hypotheses explaining the distribution of *P. clarkii* (Burnham and Anderson, 2002; McIntire and Fajardo, 2009). First, we built models relating to presence / absence of *P. clarkii* to the variables representing the four *a priori* hypotheses (Table 1). We built exploratory models considering only one hypothesis

at each time; subsequently, we built complex models representing all combinations of hypotheses. We then calculated the Akaike's Information Criterion (AIC) for each model: models explaining most of the variation with less predictors have the lowest AIC values and are considered to be the "best models". AIC may select overly complex models, therefore we considered a complex model as a candidate model only if it had AIC less than the AIC of all its simpler nested models (Richards, 2008). For each candidate model, we calculated the Akaike's weight w (AIC weight), representing the probability of the different models given the data (Lukacs et al., 2007). We estimated the amount of variation explained by models using Nagelkerke's R^2 (R^2_N). This procedure was repeated twice, with SEVMs and with GAMs; in all models, we assumed binomial error distribution. We used variance partitioning to evaluate the amount of variation explained by the environmental variables and by "spatial variables", represented by extracted eigenvector(s) in SEVM, and by the coordinates included as covariates in GAMs (Beale et al., 2010; Cushman and McGarigal, 2002).

Finally, we analysed the pattern of spatial autocorrelation, to evaluate whether it can provide information about the scale at which the invasion process occurs. We used Moran's I to assess at multiple spatial scales the spatial autocorrelation of (a) the distribution of *P. clarkii*; (b) the residuals of an ordinary least squares (OLS) logistic model relating *P. clarkii* distribution to wetland features and urban cover (the best model obtained in the results), and (c) the eigenvector extracted by the best SEVM model to reduce spatial autocorrelation (Diniz-Filho and Bini, 2005). We performed analyses using R 2.9 (www.r-project.org); we built correlograms using SAM 3.0 (Rangel et al., 2010).

Table 1. *A priori* hypotheses on processes that may explain the distribution of *P. clarkii*, and environmental variables recorded to test these hypotheses.

	Hypothesis	Environmental variables	PCA factors
1	Connectivity along hydrographic network / major waterbodies	Isolation[1] (m)	-
2	Landscape alteration	Urban cover (%)[2]	-
3	Wetland abiotic features	Maximum depth (cm)[1]	Wetland
		Surface area (m²)[1]	
		Hydroperiod (see text)	
4	Wetland vegetation features	Canopy cover (%)	Vegetation_1
		Shoreline vegetation (%)	Vegetation_2
		Sum of subemergent, emergent and floating vegetation (%)	
		Surrounding grass (%)	
		Surrounding scrub (%)	

[1]: the variable was transformed using natural logarithms.

[2]: the variable was squaretoot arcsine transformed prior to analyse.

2.3. RESULTS

We detected *P. clarkii* in 16 out of the 119 wetlands. In all wetlands where we detected its presence, we also detected the presence of juveniles, indicating that these constitute reproductive populations.

The analysis of detectability showed that, for all sites where we did not observe *P. clarkii*, the probability of occupancy was always < 0.01; for all wetlands, four surveys were always enough to detect *P. clarkii* with confidence > 95%. This indicates that our surveys estimated presence / absence with reliability.

Exploratory models

Using both SEVM and GAMs, *P. clarkii* was significantly associated with less isolated wetlands, and to large, permanent wetlands (Table 2). Furthermore, in GAMs *P. clarkii* was associated with wetlands with low scores for the variable Vegetation_2, representing an association with limited canopy cover and abundant emergent vegetation.

Table 2. Exploratory models considering only one hypothesis.

Variables	SEVM		GAM	
	χ^2_1	P	χ^2_1	P
Isolation	4.24	0.039	15.82	<0.001
Urban cover	1.943	0.163	0.789	0.375
Wetland*	11.65	<0.001	419.30	<0.001
Vegetation_1*	0.39	0.531	0.89	0.346
Vegetation_2*	0.81	0.367	20.33	<0.001

*: PCA scores. See Table 1.

Spatial Eigenvector Mapping

In all SEVMs, one eigenvector was enough to reduce spatial autocorrelation to non-significant values and was included in the models. The eigenvector best reducing autocorrelation was the same in all models with high support.

The model with the highest AIC weight suggests that the distribution of *P. clarkii* is influenced by the joint effect of wetland abiotic features and landscape alteration (Table 3a). According to this model, *P. clarkii* was significantly associated to the largest, permanent wetlands (χ^2_1 = 12.4, *P* < 0.001); although urban cover was included in this model, it did not show a significant effect (χ^2_1 = = 2.7, *P* = 0.10). This model explained a substantial proportion of variation (R^2_L = 0.42). In the best model, the SEVM eigenvector accounted for most of the explained variation (62%); wetland features were the environmental variable with the highest explanatory power, while the contribution of urbanization was limited (Table 4a). A simpler model, that did not consider urbanization, had a slightly lower support (*w* = 0.41), and explained a comparable amount of variation (R^2_L = 0.39). A candidate model considering isolation only had a very limited support (*w* = 0.01). Vegetation was not included in any candidate model.

Generalized additive models

Also with GAMs, the model with the highest weight (*w* = 0.69) was the one considering the joint effect of wetland abiotic features and landscape alteration (Table 3b). According to this model, *P. clarkii* was significantly associated to the largest, permanent wetlands within the most human-dominated landscapes (P < 0.001 for both variables). A second model, considering the joint effect of wetland features and isolation, had a lower support (*w* = 0.23; Table 3b). This second model suggests that *P. clarkii* was associated to the large, permanent wetlands less isolated from the major waterbodies. A simpler model, considering wetland features only, had a limited support (w = 0.08). All models that

excluded wetland features had very low support. Vegetation features were not included in models with high support (Table 3b). Also with GAMs, the spatial component explained most of variation; wetland features was the environmental variable with the highest contribution, while urban cover / isolation explained a minor proportion of variation (Tables 4b and 4c). Finally, it should be remarked that when using GAMs, the best models explained nearly 99% of variation, suggesting that some form of overfitting may occur.

Spatial autocorrelation

Autocorrelation of species distribution was positive and significant at distances up to 2500 m (Figure 2). OLS residuals and the eigenvector extracted by SEVM showed similar autocorrelation pattern, with generally positive and significant values at distance up to 2500 m, and negative or non significant values ad distances > 5000 m (Figure 2).

Table 3. Candidate models explaining the distribution of *P. clarkii* on the basis of environmental variables. a: Models built using spatial eigenvector mapping (SEVM); b: Models built using generalized additive models. Models are ranked according to their Δ-AIC; the model with the lowest Δ-AIC is the best AIC model.

Rank	Environmental variables	AIC	Δ-AIC	*w*
a:	Spatial eigenvector mapping			
1	Wetland features*; Urban cover, SEVM eigenvector	70.67	0.00	0.581
2	Wetland features*; SEVM eigenvector	71.38	0.71	0.406
3	Isolation, SEVM eigenvector	78.79	8.12	0.010
b:	Generalized additive models			
1	Wetland features*; Urban cover	15.36	0.00	0.691
2	Wetland features*; Isolation	17.59	2.23	0.227
3	Wetland features*	19.75	4.39	0.077
4	Urban cover; Isolation	25.75	10.39	0.004
5	Isolation	28.35	12.99	0.001
6	Urban cover; Vegetation_1*, Vegetation_2*	31.39	16.03	<0.001
7	Vegetation_1*, Vegetation_2*	33.21	17.85	<0.001
8	Urban cover	33.22	17.86	<0.001

AIC: Akaike information criterion.

Δ-AIC: difference between the AIC of each model and the AIC of the best model.

w: AIC weight of the model.

*: PCA scores. See Table 1.

Table 4. Variance partitioning representing the amount of explained variation accounted for by the best models.

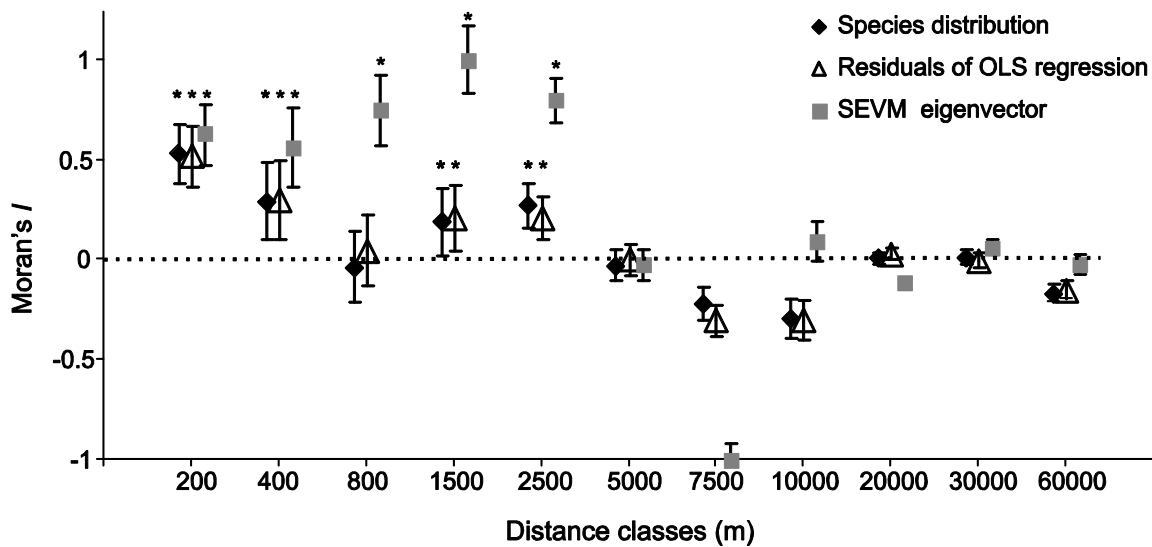| Variable	%
a: Spatial Eigenvector Mapping	
Wetland features	26.4
Urban cover	6.9
Space (SEVM eigenvector)	62.1
Joint	4.6
b: Generalized additive models, Model 1	
Wetland features	22.3
Urban cover	7.1
Space (coordinates)	63.1
Joint	7.4
c: Generalized additive models, Model 2	
Wetland features	22.7
Isolation	12.8
Space (coordinates)	53.8
Joint	10.6

Figure 2. Spatial autocorrelation (Moran's *I*), measured at multiple distance classes, of the *P. clarkii* distribution pattern (black diamonds); of residuals of OLS regression relating *P. clarkii* distribution to environmental variables (empty triangles); and of the eigenvector extracted by spatial eigenvector mapping (grey squares). Error bars represent twice the standard error of Moran's *I*; asterisks indicate that *I* was positive and significant.

2.4. DISCUSSION

Our analysis showed that multiple processes can explain the distribution of *P. clarkii* at the early stages of the invasion; two different statistical approaches (Spatial eigenvector mapping and spatial generalized additive models) yielded essentially the same results, suggesting that our conclusions are robust.

Regression models (Table 3) suggest that two processes are the most likely drivers of the distribution of *P. clarkii*: wetland suitability and alteration of the surrounding landscape. *Procambarus clarkii* is a generalist species that can occupy a wide range of wetlands, including small temporary waterbodies; it has been suggested that habitat characteristics can have only marginal significance (Cruz and Rebelo, 2007; Gherardi, 2006). Nevertheless, our analyses showed that, at the early stages of the invasion, wetland features can be extremely important for crayfish distribution: *P. clarkii* was strongly associated to large and permanent wetlands. Wetland hydroperiod is known to be a limiting factor for the presence of this species: *P. clarkii* can also be found in temporary wetlands, but in such habitats it needs shelter, like boulders, wood debris, crevices or a silt substrate to make burrows and retain the humidity, allowing their survival (Correia and Ferreira, 1995; Cruz and Rebelo, 2007; Ilhéu et al., 2003). Furthermore, wetlands should have a hydroperiod longer than 4 months for the persistence of *P. clarkii* (Gutiérrez Yurrita and Montes, 1999). Large, permanent wetlands can therefore act as major sources of *P. clarkii*, and the proximity to such wetlands is a key factor determining crayfish presence in temporary biotopes (Cruz and Rebelo, 2007). In our study, we have detected a significant effect of isolation in exploratory univariate models (Table 2), but the support of the isolation hypothesis was limited in multivariate models (Table 3). The difference between our results and the ones of Cruz and Rebelo (2007) may be related to differences in statistical modelling. In our analyses, both SEVMs and GAMs included predictors describing the spatial arrangement of data. These "spatial predictors" were extremely important in explaining crayfish distribution and accounted for most of the variation

explained by models (> 50%; Table 4). Therefore, we do not argue that proximity to invaded wetlands is unimportant, however, the analysis of spatial autocorrelation helps to explicitly assess the scale at which isolation may take effect, influencing wetland occupancy.

Spatial autocorrelation is pervasive in species distribution, and can be caused by exogenous and endogenous processes. Exogenous autocorrelation arises when species distribution is influenced by spatially autocorrelated environmental features. Endogenous autocorrelation arises through biological processes occurring at population level (e.g., aggregation, dispersal) that determine the spatial distribution of populations (Beale et al., 2010; Wagner and Fortin, 2005). Discriminating between exogenous and endogenous autocorrelation is challenging; in principle, exogenous autocorrelation can be removed from models if all relevant autocorrelated predictors are considered. Although it is always possible that some relevant, unidentified predictor is missing, the analysis of residual autocorrelation, or the analysis of "spatial predictors" removing autocorrelation (e.g., SEVM eigenvectors), can provide insight on endogenous processes determining species distribution (Chapman et al., 2009; Diniz-Filho and Bini, 2005; Dormann, 2009; Dormann et al., 2007; Van Teeffelen and Ovaskainen, 2007).

In our analysis, species distribution was positively autocorrelated at distances up to 2500 m (Figure 2); this indicates that wetlands that are less than 2500 m apart tend to have similar occupancy. In other words, the presence of occupied wetlands within 2500 m increases the likelihood of occurrence of *P. clarkii*. The pattern of autocorrelation was similar for raw species distribution data, for the residuals of OLS models and for the SEVM eigenvectors (Figure 2); the similarity of results obtained through different approaches suggests that autocorrelation may be linked to some endogenous process. Dispersal is a major source of endogenous autocorrelation (Beale et al., 2010). *P. clarkii* shows physiological and ethological adaptations allowing a remarkable dispersal ability (Barbaresi and Gherardi, 2000; Gherardi, 2006; Payette and McGaw, 2003). It may disperse both in water and overland where it can survive long periods. The overall locomotory

activity can even exceed 3 km per day (Barbaresi and Gherardi, 2000; Gherardi et al., 2002). Therefore 2500 m might correspond to the distance at which dispersal influences the distribution of populations; analyses performed in the Iberian peninsula using logistic regression yielded comparable results, and showed that the likelihood of presence of *P. clarkii* increases if there are occupied wetlands at distances ≤ 2000 m (Cruz and Rebelo, 2007). In this respect, the analysis of spatial autocorrelation may be more flexible and appropriate than logistic regression to identify such distance, because it is a spatially explicit approach while logistic regression has constraints limiting its capability to detect threshold distances (Ficetola and Denoël, 2009). Finally, dispersal is often context dependent, and individuals can cover different distances in diverse environments (Cruz and Rebelo, 2007; Olden, 2007). Lastly, natural dispersal is not the only possible explanation of the autocorrelation pattern. *Procambarus clarkii* is sometime captured by fisherman that may release it in nearby wetlands (i.e., human assisted dispersal). Furthermore, it is possible that our analyses lack relevant autocorrelated predictors that influence species distribution.

Landscape alteration was a further process important for the distribution of *P. clarkii* (Table 3). This feature may facilitate the environmental presence of *P. clarkii* through multiple nonexclusive mechanisms. First, proximity to urban areas increases the possibility that humans introduce this alien species into new wetlands. Introductions can be both accidental and deliberate, for example for food purposes or recreational scopes (e.g., release of pets, fishing) (Cruz and Rebelo, 2007; Cutway and Ehrenfeld, 2009; DiStefano et al., 2009; Hirsch, 2009). Furthermore, wetlands in altered landscapes can have simplified communities with a reduced number of species and different predators (Didham et al., 2007; Pickett et al., 2001). Generalist invasive predators with high tolerance to human disturbance, such as *P. clarkii*, can take advantage of these environmental features, reaching high densities and potentiallly disperse to more natural environments (Correia, 2003; Cutway and Ehrenfeld, 2009; Didham et al., 2007). Nevertheless, landscape

alteration explained a limited amount of variation in our models (Table 4), and the other processes are probably more important in determining invasion dynamics.

In conclusion, our analyses suggest that crayfish invasion can be favored by the colonization of large, permanent wetlands (e.g., small lakes, large ponds) in human dominated landscapes, where it can establish numerous and stable populations. These waterbodies can act as a source for the dispersal and colonization of nearby smaller, temporary or isolated wetlands (Cruz and Rebelo, 2007; Gherardi et al., 2002; Keller et al., 2008). Prevention of new introductions would certainly be the optimal strategy to limit the spread of this species (Keller et al., 2008). Unfortunately, new introductions continue: the identification of major factors determining the early stages of invasion may help to set up protocols for early monitoring and we feel that, our analyses helps to identify areas most at risk and where screening can be focused. Early detection when crayfishes are at low densities may allow for the establishment of control strategies that maintain low densities (Aquiloni et al., 2009; Aquiloni et al., 2010; Hein et al., 2007), and therefore limit the dispersal into nearby but isolated waterbodies.

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3.

Wetland features, amphibian communities and distribution of the alien crayfish *Procambarus clarkii*

ABSTRACT

Alien invasive species are a major threat to amphibians. The red swamp crayfish, *Procambarus clarkii*, is native of Eastern North America but has been introduced worldwide, and can cause dramatic declines of amphibians. We analysed the distribution of amphibians and of *P. clarkii* in an area of Northern Italy where the crayfish has been recently introduced. We assessed the relationship between wetland features, the distribution of *P. clarkii*, and the richness and structure of amphibian communities. We surveyed 114 wetlands using a combination of standard methods; we recorded environmental features (size, depth, hydroperiod), and analysed relationships using generalized additive models, including components taking into account spatial autocorrelation. We found the richest communities in wetlands with intermediate size and hydroperiod. Conversely, *P. clarkii* was associated to the largest, permanent wetlands. Amphibian communities were significantly nested; wetlands with intermediate size and hydroperiod hosted communities with less gaps than expected by chance. However, two species (*Bufo bufo* and *Salamandra salamandra*) were less nested than the other amphibians. Management focusing on relatively small, semipermanent wetlands, that are isolated from the main hydrographic network, may be an effective strategy for amphibian conservation, because these wetlands can have suboptimal features for *P. clarkii*. Nevertheless, these wetlands are not enough for the conservation of the whole amphibian community, because some species have peculiar requirements.

KEY WORDS: management, wetland suitability, hydroperiod, impact trend.

3.1. INTRODUCTION

Alien invasive species (AIS) pose major threats to conservation, as they can cause the decline of native species through multiple mechanisms, such as competition, predation, spread of diseases and complex modifications of habitats (Diamond, 1989; Smith et al., 2009; Strayer et al., 2006). Amphibians are particularly susceptible to the negative consequences of AIS; among terrestrial vertebrates, amphibians are the class for which the highest proportion of species is threatened by AIS (McGeoch et al., 2010). Invasive amphibians and fishes are the most often cited AIS determining the decline of native amphibians. For example, invasive amphibians are implicated in the spread of the chytrid fungus *Batrachochytrium dendrobatidis*, which is the aetiological agent of chytridiomycosis (Fisher and Garner, 2008; Garner et al., 2006; Soto-Azat et al., 2010); the predation by alien fishes can cause reproductive failure and the extinction of amphibian populations (Adams, 2000; Denoël et al., 2005; Knapp, 2005); invasive amphibians can threat native species through predation, competition and toxicity (Adams, 2000; Crossland et al., 2008; Kats and Ferrer, 2003; Smith, 2005).

Other exotic predators, such as crayfishes, can pose serious threats to amphibian populations, but have received a more limited attention (Gherardi, 2006). The red swamp crayfish, *Procambarus clarkii*, is native of Eastern North America and Mexico, but has been introduced for aquaculture in all continents except Australia (Huner, 2002). *Procambarus clarkii* is currently present in most countries of Western Europe; large territories have been invaded in the Iberian Peninsula, France and Italy (Gherardi, 2006). *Procambarus clarkii* can effectively prey on larvae of several species of European amphibians (Cruz et al., 2006a; Cruz and Rebelo, 2005; Gherardi et al., 2001), and the presence of this crayfish can exclude amphibians from potentially suitable reproductive areas (Cruz et al., 2006a; Cruz et al., 2006b). For instance, *P. clarkii* caused the disappearance of >50% of amphibian species from the Paul do Boquilobo Nature Reserve in Portugal (Cruz et al., 2008), and the extinction of the threatened frog *Rana latastei* from part of its small range (BERNINI et al.,

2007). Unfortunately, the complete eradication of invasive populations of *P. clarkii* is extremely difficult, and would require the application of multiple approaches (Aquiloni et al., 2009; Aquiloni et al., 2010). Intensive trapping may reduce its abundance (Hein et al., 2007), but this approach would be extremely expensive and can not be applied at large scale. On the other hand, management actions can be targeted to specific areas with the highest conservation priority, to mitigate impact. Such an approach might be more effective in areas where environmental suitability for *P. clarkii* is limited.

Hydroperiod and size are key features of wetlands, and determine suitability for amphibians (Van Buskirk, 2005; Werner et al., 2007). Small wetlands with short hydroperiod (ephemeral wetlands) have a high risk of drying before amphibian larvae attain metamorphosis. On the other hand, large wetlands with long hydroperiod (permanent) often have a high abundance of native and exotic predators that increase larval mortality. For these reasons, the richest communities of amphibians are frequently found in wetlands with intermediate size and hydroperiod (Van Buskirk, 2005; Werner et al., 2007), while only a few species adapt to wetlands with very short or very long hydroperiod (Van Buskirk, 2003). On the other hand, hydroperiod and wetland features can also influence suitability for AIS, such as *P. clarkii* (Adams, 2000; Cruz and Rebelo, 2007).

In this study, we analysed an area where *P. clarkii* was introduced only recently (about five years ago: Fea et al., 2006; unpublished data), and where it currently does not attain very high densities; therefore, well targeted management actions can be effective at this stage. We analysed the relationship between wetland features (hydroperiod; wetland size) and the distribution of both amphibians and *P. clarkii*, to evaluate whether wetland features determine suitability in a different direction for these taxa. If the maximum suitability for amphibians is attained in wetlands with different features from the ones with the highest suitability for *P. clarkii*, management efforts can be focused to the areas where suitability for amphibians is high while environmental features are suboptimal for *P. clarkii*. Furthermore, we analysed nestedness of amphibian communities. A set of

communities is nested if the species composition of species-poor sites is a proper subset of the richest ones (Patterson, 1986; Ulrich et al., 2009). In this case, poorer fragments tend to have similar communities, composed mostly by widespread species, while the conservation of richest sites may allow the maintenance of the majority of species. Therefore, the analysis of nestedness can provide important information on which species take advantages from a given management strategy (Atmar, 1993; but see also Fischer and Lindenmayer, 2005).

3.2. METHODS

Study area and sampling

We considered 114 wetlands (ponds, slow stream ditches and small lakes) in Lombardy region, Northern Italy. The study area is located North of the city of Milano, comprises the upper portion of the Po river lowland and the foothills of Brianza; the altitude range is 150-397 m (Figure 1). The study area is human dominated; agricultural and urban areas constitute the most frequent land use. Nevertheless, several natural parks (e.g., Groane regional park, Lambro Valley regional Park, Curone Valley regional park) protect natural areas and host important localities for amphibian conservation (e.g., Ficetola et al., 2009; Siesa et al., 2009a). We surveyed each wetland four times from March to June. In each survey, we used multiple techniques to assess the presence of amphibians and of *P. clarkii*. For amphibians, we used visual encounter surveys, clutch counts, repeated dip netting of wetland bottoms and banks, and audio point counts (5 min. each) to identify calling males of anurans (Dodd, 2010; Heyer et al., 1994). For *P. clarkii*, we used nocturnal visual surveys, dip netting, and the identification of exuviae (Reynolds et al., 2006) . We performed three surveys after dusk and one survey in daytime. Failing to detect a species during all sampling occasions does not necessarily indicate the species is absent, leading to the risk of underestimation of species occupancy, which may influence the outcome of

analyses (MacKenzie et al., 2006). Nevertheless, preliminary analyses of detectability performed using PRESENCE (Hines, 2006; MacKenzie et al., 2006) showed that four surveys allowed to detect *P. clarkii* and most of amphibians with high confidence (Siesa et al., 2010a; Siesa et al., unpublished manuscript). In late May-early June we measured maximum depth and surface area of each wetland. We considered a wetland temporary if it was dry or nearly dry in at least one of the surveys.
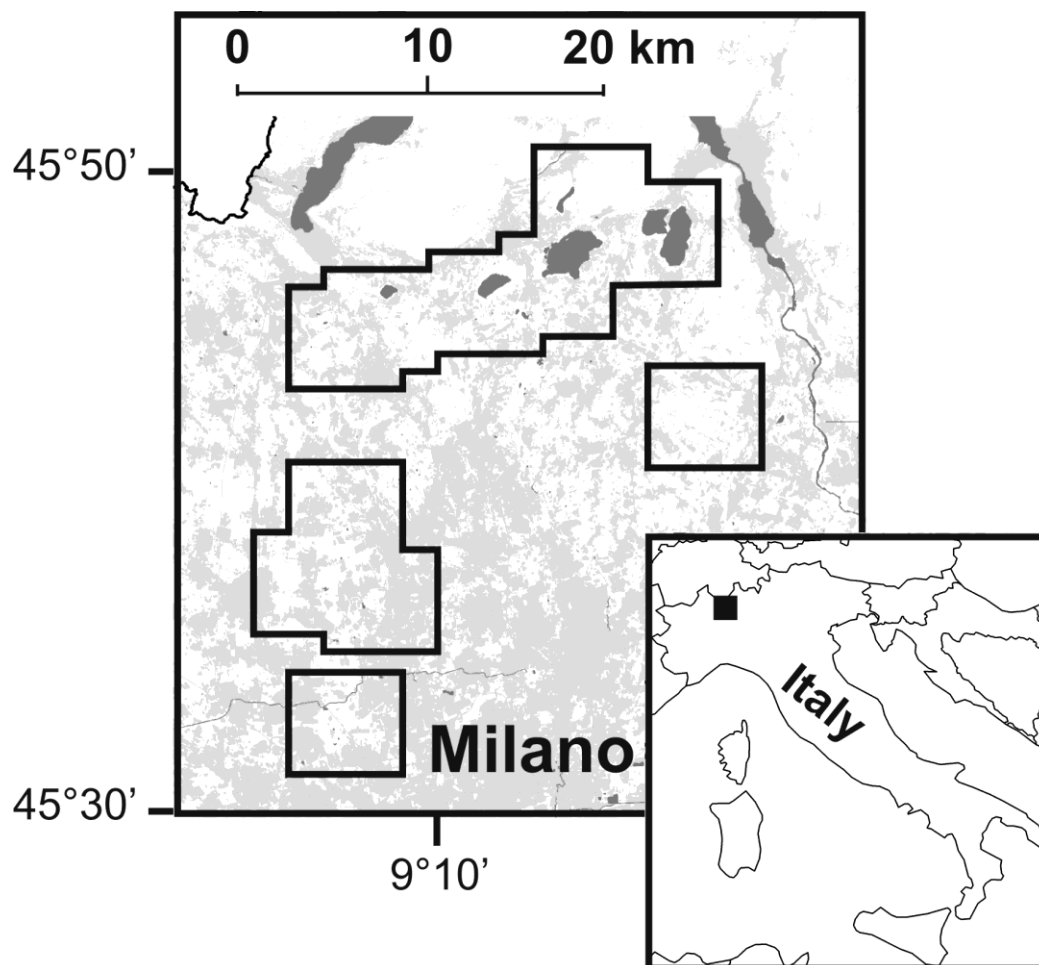

Figure 1. Study area (Lombardy region, Northern Italy). Black lines enclose the areas where wetlands are located, dark shaded areas represent lakes and major rivers, light shaded areas represent urban areas.

Statistical analyses

The three wetland features measured (area, depth and permanence) were strongly correlated among them: large wetlands were deeper and permanent. We therefore used principal component analysis (PCA) to reduce these variables to a single component representing the gradient of wetland features (see Van Buskirk, 2005; Werner et al., 2007 for a similar approach); area and depth were transformed using natural logarithms prior to run PCA, to improve normality. PCA extracted a single component explaining 50% of variation; PCA scores were positively related to wetland area ($r = 0.54$, $P < 0.001$) and depth ($r = 0.83$, $P < 0.001$), and to permanent hydroperiod ($r = 0.68$, $P < 0.001$). Therefore, PCA scores represent a gradient from small, shallow, temporary wetlands to large and permanent waterbodies.

We used generalized additive models (GAMs) to analyse the relationships between wetland features, the presence / absence of *P. clarkii*, and the richness of amphibian communities. GAMs are a semi-parametric extension of generalized linear models; the advantage of GAMS over linear models is that the shape of the response curves describing the relationships between dependent and continuous independent variables are data driven, instead of being predefined by linear or quadratic terms (Denoël and Lehmann, 2006; Wood, 2006). This flexibility allows GAMs to better fit non linear relationships with unknown features. GAMs can handle non-normal error distribution, and are particularly suited to evaluate whether ecological relationships are curvilinear (e.g., Denoël and Lehmann, 2006; Ficetola and Denoël, 2009). In GAMs, increasing values for the effective degrees of freedom (*edf*) indicate increased complexity and non-linearity of the response curve (Wood, 2006). We therefore identified a clearly nonlinear response if *edf* > 2 (Ficetola and Denoël, 2009). For logistic models (i.e., models with binomial error), the term "linearity" was referred to the plot on the logit scale (Ficetola and Denoël, 2009).

Our data have a strong spatial structure. Spatially structured data need to be analysed using appropriate statistical methods, because the presence of spatial

autocorrelation may bias the results of standard regression techniques (Beale et al., 2010; Dormann, 2007). We therefore incorporated the coordinates of data points as covariates in GAMs, following the approach detailed by BEALE et al. (2010). Simulation studies showed that this is among the techniques with the best performance in the analysis of spatial data; this approach has limited bias even in the presence of violations of assumptions, such as non-stationariety of autocorrelation (Beale et al., 2010). In our analysis, we used a binomial error distribution to assess the relationship between the presence / absence of *P. clarkii* and wetland features; we used Poisson error distribution to assess the relationship between species richness and wetland features; we used likelihood ratio tests to assess significance. We performed the analyses in R (www.r-project.org).

We used the metric NODF (Nestedness metric based on Overlap and Decreasing Filling) to assess the nestedness of the system of amphibian communities (Almeida-Neto et al., 2008). Communities and species are represented by a matrix in which each row is a site, and each column is a species. The NODF metric ranges from 0 to 100, and can be defined as the percentage of species in right columns, and communities in inferior rows overlapping, respectively, with those found in left columns and upper rows; nested matrices have higher values of NODF (Almeida-Neto et al., 2008; Ulrich et al., 2009). NODF has advantages over other metrics of nestedness, such as the possibility to assess separately nestedness of species and sites, and the possibility to order communities following explicit ecological gradients; furthermore, NODF is less prone to statistical errors than other metrics (Almeida-Neto et al., 2008). Sorting the communities according to ecological factors allows to test hypotheses on the causes of nestedness of the system (Guimaraes and Guimaraes, 2006; Ulrich et al., 2009). Therefore we sorted sites according to the scores obtained from GAMs relating richness to wetland features and we sorted species in decreasing order of frequency. We assessed significance of nestedness using 1000 permutations of random matrices; we used the null model CE to produce random matrices. In this null model, the probability of occupancy of a cell $a_{i,j}$ is $(P_i/C + P_j/R)/2$, where P_i is the number of presences in a row i, P_j is the number of presences in a column j,

C and *R* are the number of columns and rows, respectively (Guimaraes and Guimaraes, 2006). Analyses performed using different metrics and null models, such as ATMAR & PATTERSON's (1993) *T,* yielded equivalent results (not shown). We also calculated nestedness individually for species, to identify species with less nested distribution (Almeida-Neto et al., 2008). We performed nestedness analyses using ANINHADO (Guimaraes and Guimaraes, 2006).

3.3. RESULTS

In our surveys we detected *P. clarkii* in 12 wetlands. Furthermore, we detected the presence of nine amphibians: the fire salamander *Salamandra salamandra*; the Italian crested newt *Triturus carnifex*; the smooth newt *Lissotriton vulgaris*; the common toad *Bufo bufo*; the Italian tree frog *Hyla intermedia*; the agile frog *Rana dalmatina*; the Italian agile frog *R. latastei*; the common frog *R. temporaria* and the pool frog *Pelophylax* synklepton *esculentus*. 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. Species richness per wetland ranged from 0 to 9 (average ± SE:2.01 ± 0.14).

We observed *P. clarkii* in wetlands with a large range of environmental features, ranging from small lakes (e.g., Lake Alserio) to about 20 m^2 ponds. Nevertheless, the distribution of *P. clarkii* was strongly related to positive scores of the PCA component describing wetland features (GAM: χ^2 = 616.5, *edf* = 1, *P* < 0.001). The relationship between suitability for *P. clarkii* and PCA scores was strictly linear (*edf* = 1), indicating that suitability steadily increased in large, deep wetlands with permanent hydroperiod (Figure 2a).

The richness of amphibian communities was significantly related to the PCA component describing wetland features (GAM: χ^2 = 7.94, *edf* = 2.2, *P* = 0.023). However, the relationship between hydroperiod and richness of amphibian communities was non

linear (*edf* = 2.2). We observed the richest communities in wetlands with intermediate PCA scores, indicating that suitability is highest in wetlands with intermediate size and hydroperiod (Figure 2b). When taking into account wetland features, the relationship between the richness of amphibian communities and the presence of *P. clarkii* was not significant (GAM: χ^2 = 0.47, *df* = 1, *P* = 0.49).

When sorted following the GAM scores, the community set was significantly nested (Figure 3). NODF of the system was 33.9, and was significantly higher than nestedness of random matrices (average NODF = 20.6; range: 14.3-26.7, permutation *P* < 0.001) (Figure 3). The average nestedness of species was 44.3. However, two species showed considerably lower nestedness: *S. salamandra* (NODF = 25.8) and *B. bufo* (NODF = 21.0) (Figure 3).

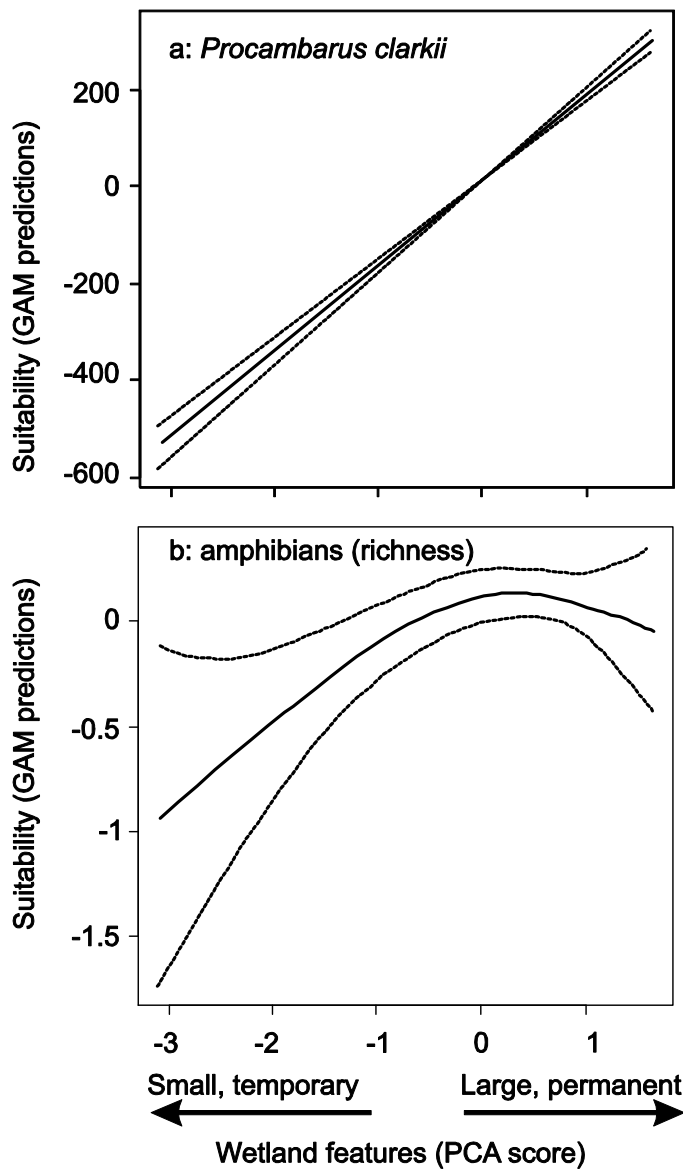**Figure 2.** Response curves of generalized linear models, describing the relationship between pond features (components extracted by Principal Component Analysis) and (a) presence of *Procambarus clarkii*; (b): richness of amphibian communities. Dotted lines represent 95% confidence intervals. The y-axes are based on residuals, and indicate the influence of the explanatory variable on the prediction.
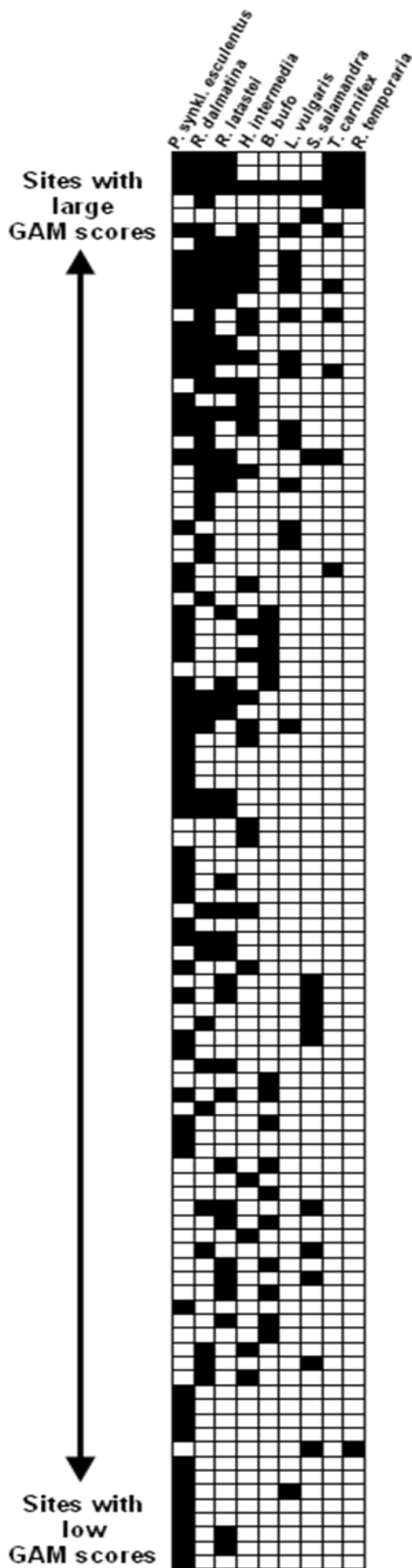
Figure 3. Matrix of species distribution; each row represents one site, each column represents the distribution of one species. Communities are sorted following score of GAMs. Only sites with at least one species of amphibian are represented.

3.4. DISCUSSION

Our study showed that amphibian communities and the alien crayfish *Procambarus clarkii* are strongly influenced by wetland features (size, depth and hydroperiod), but the shape of the relationships was different between amphibians and *P. clarkii*. For both taxa, the smallest, temporary wetlands had very low suitability. However, for *P. clarkii* suitability was highest in the largest, permanent waterbodies, while amphibian species richness was highest in wetlands with intermediate size and hydroperiod (Figure 2). The curvilinear relationship between PCA scores and community richness was not unexpected; other studies have found the highest amphibian richness in wetlands with intermediate features (e.g., Van Buskirk, 2005; Werner et al., 2007). This likely occurs first because only species with fast larval development can successfully reproduce in ephemeral wetlands (such as, in the study area, *H. intermedia* and the toad *Bufo viridis*, not detected in the visited wetlands) (Ficetola and De Bernardi, 2004; Skelly et al., 1999; Van Buskirk, 2003), therefore these wetlands tend to have poor communities. Secondly, large permanent wetlands often have high abundance of predators (both fishes and invertebrates) (Baber et al., 2004; Werner et al., 2007); in these wetlands amphibians are often limited to the species capable to withstand predators, such as the common toad (which have larvae unpalatable to fishes: Van Buskirk, 2003) or pool frogs (which have behavioural adaptations: Semlitsch and Reyer, 1992). Therefore, wetlands with intermediate features were suitable for the majority of species, and harbour both common and rare species. For instance, GAMs predicted that the highest amphibian richness would be attained in wetlands with PCA scores close to zero (Figure 2b), which correspond to small, shallow ponds with a surface of about 70 m² and a maximum depth of about 30 cm.

The relationship between wetland features and suitability was different for the crayfish, as suitability was highest in the largest, permanent wetlands (Figure 2a). *Procambarus clarkii* can survive also in temporary wetlands, where it needs shelters or a soft substrate to dig burrows retaining humidity (Cruz and Rebelo, 2007; Ilhéu et al., 2003);

nevertheless, a longer hydroperiod can allow a longer activity period. Furthermore, *P. clarkii* can not reproduce successfully if the hydroperiod is too short (Gutierréz-Yurrita and Montes, 1999). Therefore, large permanent wetlands, particularly if non isolated or connected through the hydrographic network to other waterbodies, suffer the highest risk of invasion (Cruz and Rebelo, 2007) see also the chapter 2 of this thesis.

After taking into account wetland features, invaded water bodies did not host a lower species richness that those without *P. clarkii*. However, this does not mean that *P. clarkii* does not affect amphibian communities: first, the crayfish invaded the area recently, and the invasion can require some years until the decline of amphibians becomes noticeable (Cruz et al., 2008). Analyses of survival or reproductive success can help to understand whether amphibians suffer limited fitness in invaded wetlands, and which life history stages are more affected. Second, vulnerability can vary among species (Cruz et al., 2006a); analyses focusing on individual species, such as the ones more affected by crayfish predation or those with high conservation concern, can provide a more detailed picture.

When sorted according to GAM scores, the community set was significantly nested. This indicates that sites with high GAM scores (i.e., intermediate wetland features) host not only the richest communities, but also communities with less gaps (i.e., more complete communities) than expected on the basis of the simple variation of species richness. This further confirms the importance of these wetlands for the conservation of amphibian communities, as some of them hosted all or nearly all the amphibian species of the study area (Figure 3). Nevertheless, it should be remarked that the system was not perfectly nested, as the actual NODF score was well below 100 (which is the maximum NODF value and indicates perfect nestedness). Therefore, it is important considering wetlands with a range of different features (Fischer and Lindenmayer, 2005). Particular attention should be played to idiosyncratic species, i.e., those with less nested distribution; these species would not be protected by management efforts focusing on the conservation of the wetlands with features suitable for the majority of species (Fischer and Lindenmayer, 2005). Idiosyncratic species are often characterized by particular environmental

requirements (Ulrich et al., 2009). In this study system, two species showed a much lower level of nestedness compared to the overall community: fire salamander and common toad. This can be explained by the peculiar features of environments where they breed: the fire salamander breeds mostly in small streams, while all the other species prefer ponds (e.g., Ficetola et al., 2009); the common toad is usually associated with permanent, large waterbodies, while it is rarely found in temporary wetlands (e.g., Van Buskirk, 2005).

As *P. clarkii* invaded only recently the study area, and we did not a strong relationship between crayfish distribution and richness of amphibian communities, our study can be considered as "year zero" data. Integrating studies on alien species within a temporal framework can be extremely useful (Strayer et al., 2006). Year zero data can be used for comparisons, to assess temporal trends of both native and invasive species and to understand when the amphibian decline takes effect. For example, Cruz et al. (2008) showed a collapse of amphibian communities about ten years after the invasion of *P. clarkii*. In our study area, the crayfish was introduced about five years ago, and several wetlands have been invaded only in the last few years. This would suggests that crayfish requires some years to reach the high densities determining the amphibian decline, and / or that amphibians can survive for some period after the invasion. For instance, adults may continue to attempt breeding in invaded wetlands, even if crayfish predation determine a limited fitness. Crayfish abundance is relatively high in several of the invaded wetlands (mean: 7.7 individuals / m²; unpublished data), but is lower in the areas invaded more recently. However, the density of *P. clarkii* is expected to increase with time, and waterbodies with very high crayfish densities can also act as sources and determine the colonization of nearby waterbodies with suboptimal features (e.g., temporary wetlands). This will probably increase impact on amphibians, and might even modify the relationship between hydroperiod and crayfish distribution (Figure 2a).

Our results can have important consequences for wetland management aimed at amphibian conservation in these human dominated landscapes. In environments that are suitable for *P. clarkii*, this crayfish can cause the extinction of many amphibian species: if

crayfish eradication is not feasible, the conservation of many amphibians would depend on the existence of temporary wetlands with suitable features, isolated from the invaded waterbodies (Cruz et al., 2008). First, the preservation of existing temporary waterbodies would be extremely important. Small, temporary wetlands are sometimes overlooked in conservation plans, and are declining worldwide because they are often drained, or transformed in permanent waterbodies (for example, as water reservoirs). (Beja and Alcazar, 2003; Moser et al., 1996). Furthermore, it may be possible to actively manage the hydroperiod of wetlands, to match the requirements of target species. In extreme situations, such as the human dominated landscapes considered in this study, amphibian conservation may strongly depend on the active management of the features of landscapes and wetlands. Nevertheless, it should be remarked that temporary wetlands can not be considered an optimal solution working for all species. In these environments, amphibians sometimes attain lower fitness (Karraker and Gibbs, 2009), and species requiring long periods for metamorphosis (e.g., crested newts) can not successfully reproduce there. Networks of wetlands with different hydroperiod, allowing the persistence of species with different requirements, can be important for the maintenance of the whole communities (Beja and Alcazar, 2003; Snodgrass et al., 2000; Van Buskirk, 2003). In the last years, it is increasingly recognised that isolated, non-permanent waterbodies can play a pivotal role for amphibian conservation, as they harbour unique communities and may suffer a lower impact of AIS. In human dominated landscapes the abundance of AIS is quickly augmenting and the role of temporary wetlands for conservation is expected to increase in the next future.

4.

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

ABSTRACT

Early assessment of the impact of alien invasive species is crucial to set up timely management, but often the impact is evident when 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, and assessed whether considering measures of reproductive success provide a more prompt measure of impact than considering just species distribution. We surveyed 125 wetlands in Italy, in an area recently invaded by *P. clarkii,* 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 (generalized linear models and spatial eigenvector mapping) and community level (constrained redundancy analysis). When considering the distribution of breeding sites, only two amphibians (*Lissotriton vulgaris* and *Hyla intermedia*) where negatively related to *P. clarkii*, while the relationship between the distribution of other the species and *P. clarkii* was positive or not significant. However, for all amphibians, the abundance of larvae was negatively related to the alien crayfish. Analyses performed at community and single species levels yielded consistent results. *Procambarus clarkii* impacts amphibians through different processes. Newts probably select uninvaded wetlands for breeding; other species (e.g., frogs) attempt breeding in wetlands with crayfish, but suffer very low success because of strong predation. Considering distribution data only would not provide a correct picture of the impact of this alien species; measures of reproductive success may allow a more prompt assessment of the impact.

KEYWORDS: amphibian decline, biological invasions, breeding success, breeding habitat, landscape composition, *Procambarus clarkii*, spatial autocorrelation.

4.1. INTRODUCTION

Alien Invasive Species (AIS) are a major cause of biodiversity loss at the global scale. The prevention of introduction events, and the control of established species, are key management tools for biodiversity conservation. Management of AIS have 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 have negative consequences (Jeschke and Strayer, 2005; Vilà et al., 2010). As thousands of species have been and continue to be released, but only some of them will negatively affect biodiversity, management is often focused on those species with the strongest impact on biodiversity, or in the areas where these species have the worst consequences (Vilà et al., 2010). The rapid identification of the effects of AIS on native biota is therefore a necessary first step for management actions.

However, an early assessment of the effects of AIS may be complex for multiple reasons. In the long term, negative consequences of AIS are expected to determine modifications of 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 AIS are determining declines (e.g., Bos et al., 2008; Cruz et al., 2008; Elliott et al., 2010; Strayer et al., 2006). However, distribution data can provide an incomplete picture, because species may survive under suboptimal conditions for relatively long periods, depending on generation time. Therefore, documenting declines and extinctions can require long periods (Strayer et al., 2006), and conclusive results may be attained only when it is too late for effective management. Measuring the impact of AIS on fitness parameters of native species may allow a more prompt indicator of processes ongoing, and on the actual consequences of AIS.

Complex relationships between environmental features and species distribution constitute a further issue to understanding the impact of AIS. 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 AIS (Didham et al., 2007). As both native and AIS depend from the same environmental features, it may be difficult to establish whether the decline of a species was caused by AIS or by environmental modifications: negative relationships between native and invasive species can be observed both if AIS actually have negative effects, and if native and alien species respond differently to the same environmental modifications. Analyses considering both habitat modifications and AIS are needed to tease apart their relative role. Nevertheless, only a small subset of studies considered the effects of AIS 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 worldwide and is currently invasive in wide areas of Europe (Capinha and Anastácio, 2011; Gherardi and Panov, 2009). *Procambarus clarkii* is a keystone AIS 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 and Panov, 2009), it feeds on aquatic stages of amphibians and can cause the decline of several amphibian species (Cruz et al., 2006a; Cruz et al., 2006b; Cruz et al., 2008; Gherardi et al., 2001), 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 only recently (about five 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 amphibians 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 in wetlands where we observed breeding activity, as a measure of breeding success. We show that the two analyses offer a different perspective on the consequences of AIS on native amphibians, and that the analysis of breeding success may allow a more prompt and appropriate assessment of the effects on some native species. Each analysis was performed at two levels: single species and whole community. Single species analysis help to identify more precisely the issues of target species, community analyses can allow to unravel more complex and general relationships.

4.2. METHODS

Study area and methods outline

We considered 125 wetlands (slow stream ditches, ponds and small lakes) in Lombardy region, Northern Italy. The study area comprises the upper portion of the Po river lowland and the Brianza foothills; the altitude range is 150-397 m, see Ficetola et al. (2011c) or chapter 3 for a map of the study area. The study area is human dominated; agricultural and urban areas constitute the most frequent land use. Nevertheless, there are several natural parks protecting 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 AIS 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 related to environmental features (e.g., vegetation, climate, landscape composition) which are in turn 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 where surveyed in 2009, 11 further wetlands were surveyed in 2010 (total: 125 wetlands). During each survey, we used a combination of multiple techniques to assess the presence of breeding amphibians and of *P. clarkii*. For amphibians, we used visual encounter surveys, clutch counts, and repeated dip netting of wetland bottom and banks to assess the presence of individuals; 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 (Reynolds et al., 2006). We performed at least three surveys after dusk and at least one survey in daytime. Previous analyses showed that these methods allow a successful characterization of both breeding amphibians and *P. clarkii* (Sewell et al., 2010; Siesa et al., unpublished manuscript) and see chapter 2. 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 (Skelly and Richardson, 2010). In this period, larvae of all amphibian species are still in the breeding wetlands. Samples were collected by quickly thrusting the pipe through the water column and into the sediments, to seal the sample area. Small nets (1 mm mesh size) were used to remove all animals from

the water column and from the first centimeters of the sediment. Net sweeps were collected until at least 10 consecutive sweeps were performed without capturing any animal (Skelly and Richardson, 2010; Werner et al., 2009). We collected samples with a 0.25 m^2 circular pipe sampler. Number of pipe samples was proportional to wetland surface (average: four samples per wetland); in most of wetlands we collected four to six samples, a smaller number of samples was collected in a few, very small wetlands. We used detectability analyses (see below) to assess the reliability of this sampling. In order to maximise the possibility to detect relationships between amphibians and *P. clarkii*, pipe sampling was focused on a non-random subsample of sites. We performed pipe sampling on 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 signs of breeding activity of newts. Larvae where identified following standard keys (Lanza et al., 2007). There is morphological overlap between tadpoles of *R. dalmatina* and *R. latastei*, and they can not be identified in the field with certainty (Barbieri et al., 2000). Therefore, tadpoles of these two species were pooled in a single group ("brown frogs") for some analysis. Tadpoles of these closely related frogs have a very similar ecology (Lanza et al., 2007); it is thus likely that they respond in a similar way to *P. clarkii*. 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 recorded eight parameters used as standard characterization of amphibian breeding wetlands: maximum width, maximum depth, average stream velocity, canopy cover, percentage of riparian vegetation along the shoreline, percentage of emerging or floating vegetation on wetland surface, presence of fishes, water

permanence (Table 1). Parameters were recorded using standard bio-assessment protocols; details on their measurement are reported elsewhere (Barbour et al., 1999; Ficetola et al., 2011b, see Table 1; Van Buskirk, 2005). 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 (www.cartografia.regione.lombardia.it). We considered three land cover typologies (Table 1): urban, agriculture and natural vegetation. Land cover was measured in a radius of 100 m and of 400 m from each wetland, because previous analyses showed that amphibians respond strongly landscape features at these scales within the study area (Ficetola et al., 2009).

Statistical analyses

Species abundance obtained through pipe sampling and environmental variables were transformed using logarithms or square-root arcsin to improve normality (Table 1). Some measures of wetland morphology (area, depth, hydroperiod) and of landscape composition were strongly correlated (Pearson's correlation: $|r|$ ranging from 0.3 to 0.8); strong multicollinearity among independent variables may bias regression analyses (Berry and Feldman, 1985). To remove multicollinearity, and to reduce 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 related to area ($r = 0.62$), depth ($r = 0.83$) and hydroperiod ($r = 0.65$) ($P < 0.001$ for all correlations). Therefore, high values of abiotic features indicate large, deep and permanent wetlands. Similarly, we ran PCA over the variables describing landscape composition (percentage of wooded, urban and agricultural landscape; Table 1); we repeated this analysis at two spatial scales (100 m and 400 m of radius, see above). The component extracted by the PCA at the 400 m radius (landscape 400) explained 63% of variation of variables, and was positively related to

urban (r = 0.76) and agricultural cover (r = 0.58), and negatively related to natural vegetation (r = -0.99) at this scale (P < 0.001 for all correlations). Similarly, the component extracted by the PCA at the 100 m radius (landscape 100) explained 57% of the original variables, and was positively related to urban (r = 0.68) and agricultural cover (r = 0.72), and negatively related to natural vegetation (r = -0.99) at this scale (P < 0.001 for all correlations). After the substitution of variables with PCA scores, we found no strong correlation among environmental variables ($|r|$ ≤ 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 the species is absent; this can lead to a underestimation of occupancy and might affect the results of analyses. We used PRESENCE 2.4 to estimate occupancy, and evaluate the probability of occupancy of sites were we did not detect species (Hines, 2006; MacKenzie et al., 2006; Sewell et al., 2010). Occupancy modelling uses data on presence/absence at multiple sampling occasions, and estimates both occupancy and detection rates. It is also possible include site and survey-specific parameters as covariates (MacKenzie et al., 2006; Sewell et al., 2010). We repeated this analysis for both breeding sites data, and for pipe sampling data.

For the breeding sites dataset, we assumed that the probability of detection of each 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 and 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-run species distribution models (see below), using the probability of occupancy at a given site (as estimated by PRESENCE models) as dependent variable. Occupancy models where run separately for sites surveyed in different years.

For the pipe sampling dataset, we run occupancy models assuming that the detection probability of larvae of each species was constant across all the pipe samples collected in a given site, as all samples at a given wetland were collected during the same sampling occasion.

Species distribution

First, we analysed separately 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 and Anderson, 2002), to identify the combination of variables best describing the distribution of each species detected in >10% of sites, following the procedure detailed in Denoël *et al*. (2009). We built generalized linear models (GLM) assuming binomial error including all possible combinations of environmental variables. For each species, we performed preliminary analyses to assess at which spatial scale (100 or 400 m) it is more strongly related to landscape features, and we considered the relevant scale only (see Ficetola et al., 2009). 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 for the identification of candidate models. We considered a complex model only if it had a Δ-AIC less than the Δ-AIC of all its simpler nested models; furthermore, we considered only models including significant variables (Denoël et al., 2009; Maggini et al., 2006; Raffalovich et al., 2008; Richards, 2008). 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, given the data (Lukacs et al., 2007). Furthermore, if the presence of *P. clarkii* was included in the best models, we compared models including the same environmental variables, with and without crayfish presence; similarly, if the presence of *P. clarkii* was not included in the best model, we compared the best model with a similar model considering also *P. clarkii*. We also report significance values of variables, to facilitate the interpretation of models and of the role of environmental variables (Stephens et al., 2007).

Spatial autocorrelation may bias results of standard regressions models, therefore we used a Monte Carlo procedure to assess whether the residuals of best models were spatially autocorrelated (Lichstein et al., 2002). 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; Griffith and Peres-Neto, 2006; Peres-Neto and Legendre, 2010 for details). This implementation of SEVM is considered among the most robust spatial methods (Bini et al., 2009; Dormann et al., 2007); advantages of SEVM include the possibility to handle non-normal errors; furthermore, unlike other methods, its aim is removing residual autocorrelation (Dormann et al., 2007).

Second, we used constrained redundancy analysis (RDA) to assess the relationship between the composition of amphibian communities, environmental features and the distribution of *P. clarkii*. RDA is a multivariate, 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 datasets representing independent variables (e.g., environmental variables) (Legendre and Legendre, 1998). Spatial autocorrelation may affect also canonical analyses, therefore we integrated autocorrelation in RDA using an approach similar to the one developed by Peres-Neto and Legendre (2010). 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 and Legendre, 2010). To assess the relationship between amphibian communities and environmental features, we used a RDA considering 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 used a RDA with community composition as dependent, 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 ANOVA-like permutation tests (10,000 permutations) (Legendre and Legendre, 1998).

Larval abundance

We used GLMs to assess the relationships between the abundance of amphibian larvae and *P. clarkii* . First, to take into account the potential effect of environmental features, we built models relating larval abundance to environmental variables. In these models ('environment-only'), we included the environmental variables selected by the best models of the analysis of species distribution (see above). Subsequently, we built models considering both environmental features and the presence of *P. clarkii* ('environment+*P. ckarkii*'). For each species, models with and without *P. clarkii* were compared using AIC, as described above. We also assessed whether the environment + *P. clarkii* models performed significantly better than the environment-only models. If residuals of GLMs were spatially autocorrelated, we used SEVM instead than standard GLM. We considered the mass of *P. clarkii* (g / m^2) as a measure of its abundance, because large individuals can exert higher predation pressure. Analyses performed using the number of individuals / m^2 yielded identical results (not shown). The abundance of *P. clarkii* and of amphibian larvae was log-transformed to improve normality. We built univariate models for all species detected in >

15% of sites. One of these species (*Triturus carnifex*; see results) was not included in the species distribution model. For this species, the 'environment-only' model included a constant only, while the 'environment + *P. clarkii*' model included crayfish abundance only. Preliminary 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 detected in more than one site), environmental features and abundance of *P. clarkii*, while taking into account the effect of spatial autocorrelation (Legendre and Legendre, 1998; Peres-Neto and Legendre, 2010, see above for details). We performed all analyses using packages SPDEP and VEGAN within the R statistical environment (www.r-project.org).

Table 1. Environmental variables recorded. Some groups of variables have been summarised by factors extracted through principal component analyses (PCA) prior to perform single species or community analyses.

Environmental features	Summarized by PCA?
a: wetland features	
Surface[1] (m)	Abiotic features
Maximum depth[1] (cm)	Abiotic features
Permanence during the study period (Y/N)	Abiotic features
Stream velocity[1] (m/s)	
Fish presence (Y/N, visual estimate)	
Canopy cover (%, visual estimate)[2]	
Emerging vegetation (% of vegetation emerging from water surface,visual estimate)[2]	
Riparian vegetation (%,visual estimate)[2]	
b: landscape features	
Forest cover (%) [2, 3]	landscape 400 or landscape 100
Cropland cover (%) [2, 3]	landscape 400 or landscape 100
Urban cover (%) [2, 3]	landscape 400 or landscape 100

1: Log-transformed prior to analyse.

2: square-root arcsin transformed prior to analyse.

3: measured in a radius of 400 m for the analysis of *S. salamandra*, and in a radius of 100 m for the community analysis (Ficetola et al., 2009).

4.3. RESULTS

In our surveys we detected *P. clarkii* in 19 wetlands (occupancy, O = 15%). We detected 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.

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, 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 most likely crayfish negatively affects newt distribution. 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 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 that it was associated 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 variation explained 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 (see above). Therefore, we repeated analyses using occupancy estimated by PRESENCE as dependent variable (see Table S1 in Supporting Information). The best models where 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 2. Relationships between species distribution, environmental features and presence of *P. clarkii*: comparison of models with and without the effect of *P. clarkii*. 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			sign	χ^2	P	R^2_N	K	AIC	*w*	K	AIC	*w*
							Model including *P. clarkii*			Without *P. clarkii*		
Lissotriton vulgaris		*P. clarki* (-)	-	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 eigv.		32.1	<0.001							
Hyla intermedia	a	*P. clarki*	-	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 eigv.		20.4	<0.001							
	b	*P. clarki*	-	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 eigv.		25.7	<0.001							
Pelophylax s. *esculentus*		*P. clarki*	+	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							

Table 3. Relationships between species distribution, environmental features and presence of *P. clarkii*. The table includes species for which the presence of *P. clarkii* was nor 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.

Species		Variables	sign	χ^2	P	R^2_N	K	AIC	w	χ^2_1	P
						Best AIC models				Effect of *P. clarkii*	
Bufo bufo		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						
Rana dalmatina		riparian vegetation	+	19.6	<0.001	0.51	3	116.9	1	2.7	0.104
		SEVM eigenvectors		33.0	<0001						
Rana latastei	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 presence	+	5.8	0.016						

Species distribution: community analysis

RDA showed that, after controlling for the effect of spatial autocorrelation, community structure was strongly and significantly related to environmental features (permutation test, $P < 0.0001$; Figure 2a). Relationships between amphibians and environmental features were similar to the ones depicted by univariate models (Table 2, Figure 1a). After taking into account the effect of spatial autocorrelation and environmental features, amphibian communities were significantly related to the distribution of the alien crayfish (permutation test, $P = 0.0009$; Figure 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 (Figure 2b). Variance partitioning showed that environmental variables accounted for 40% of explained variation in community structure, spatial autocorrelation accounted for 38%, 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*: $P = 0.0003$; Figure S1 in Supplementary Material).

Distribution of larvae

We detected *P. clarkii* in 50% of wetlands surveyed with 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 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 related to the abundance of crayfish

(Figure 2, Table 4). For all species, the model 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).

It should be remarked that the relationship between larval abundance and the alien crayfish was negative and significant also for pool frogs (Table 4, Figure 2d), despite breeding sites surveys indicated an 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 related to the abundance of *P. clarkii* ($F_{1,19}$ = 4.9, *P* = 0.039; regression model taking also into account landscape and abiotic features). *Procambarus clarkii* was present in 39% of these 23 wetlands.

RDA showed that, after taking into account spatial autocorrelation and environmental features, larval communities were significantly related to the distribution of the alien crayfish (permutation test, *P* = 0.012). For all species, the abundance of larvae was negatively related to crayfish abundance (Figure 3). 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.
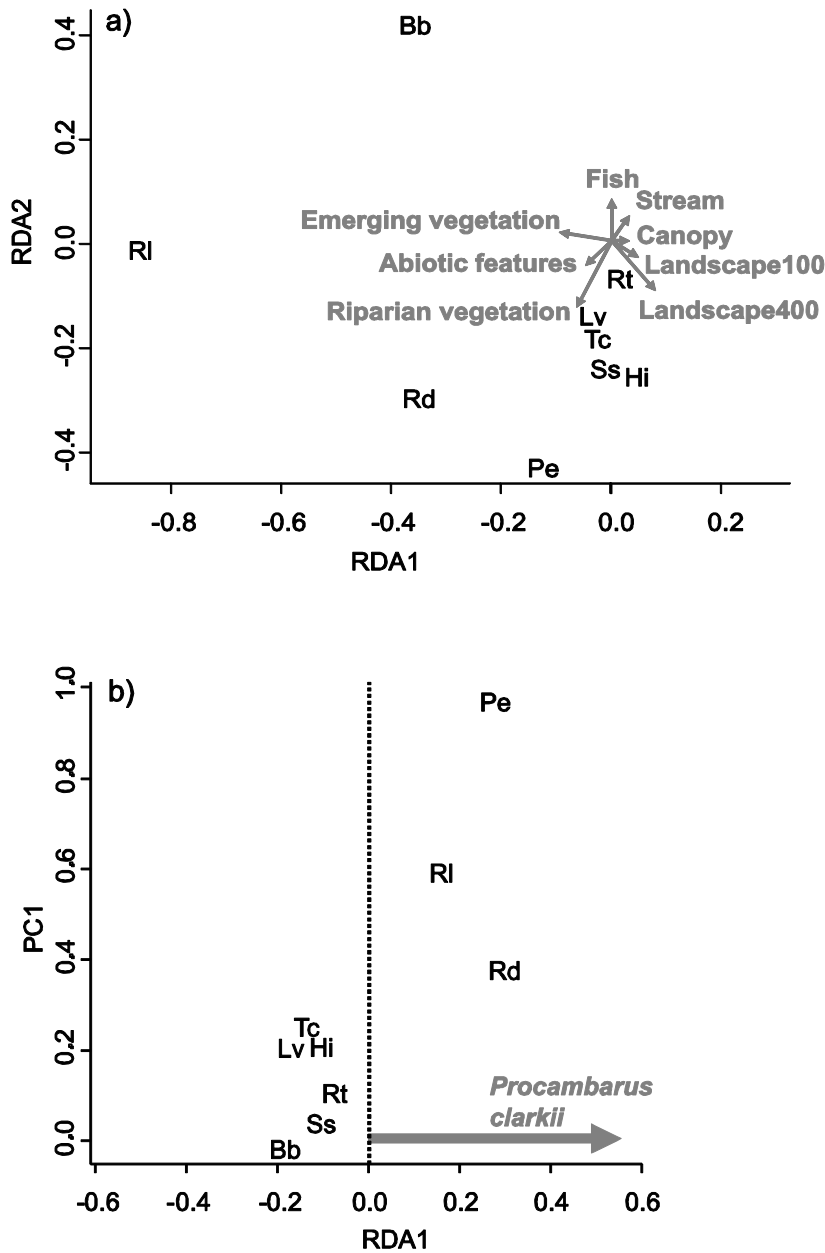
Figure 1. Results of constrained redundancy analysis showing (a) the relationship between wetland features and amphibian distribution, while taking into account spatial autocorrelation; (b) the relationship between amphibian distributing and presence of *Procambarus clarkii*, while taking into account wetland features and autocorrelation. Ss: *Salamandra salamandra*; Tc: *Triturus carnifex*; Lv: *Lissotriton vulgaris*; Hi: *Hyla intermedia*; Bb: *Bufo bufo*; Rd: *Rana dalmatina*; Rl: *R. latastei*; Pe: *Pelophylax* s. *esculentus*. Constraining variables are in grey colour.
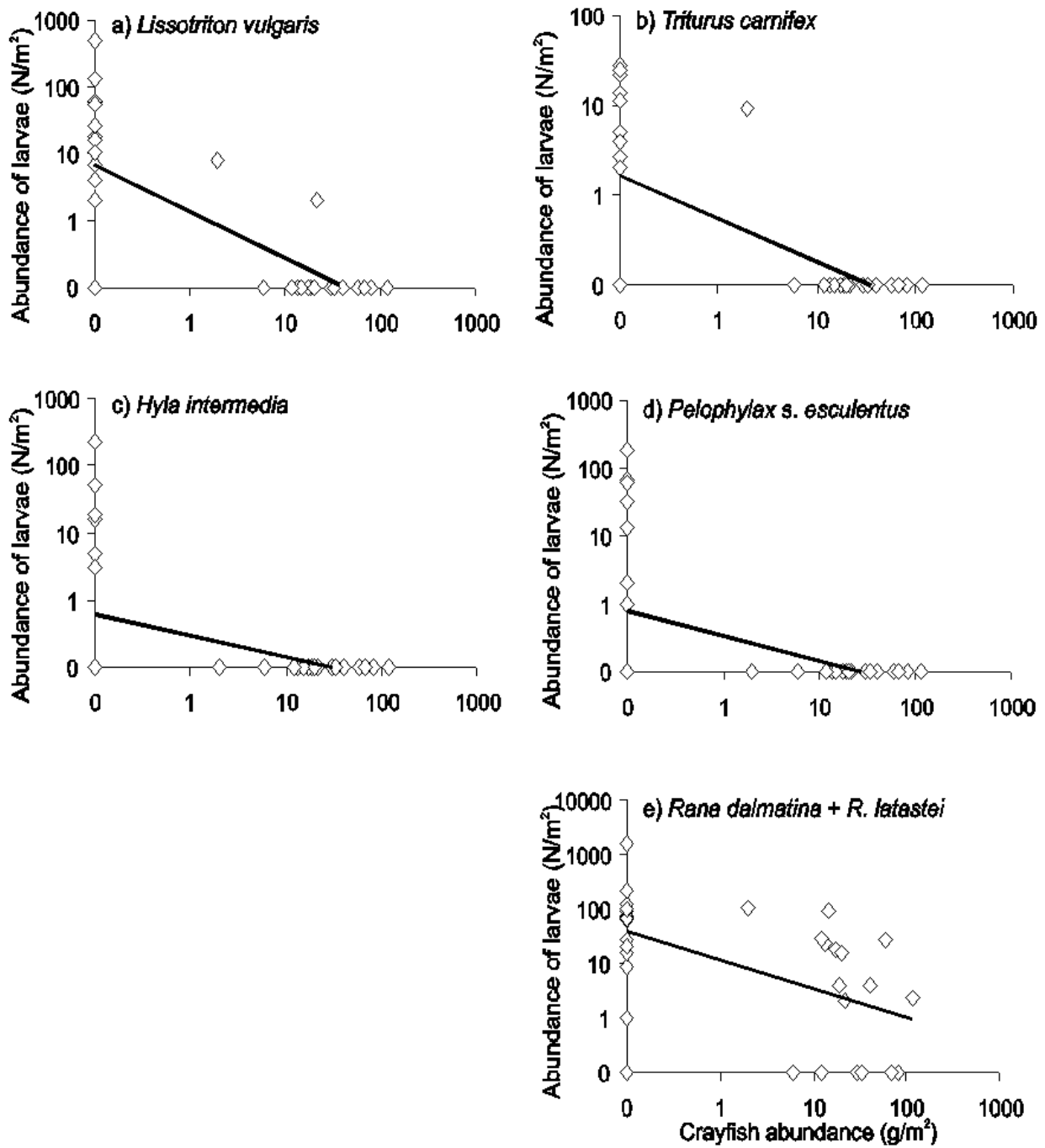
Figure 2. Relationship between the abundance of the invasive crayfish, *P. clarkii*, and the abundance of amphibian larvae in 34 wetlands.
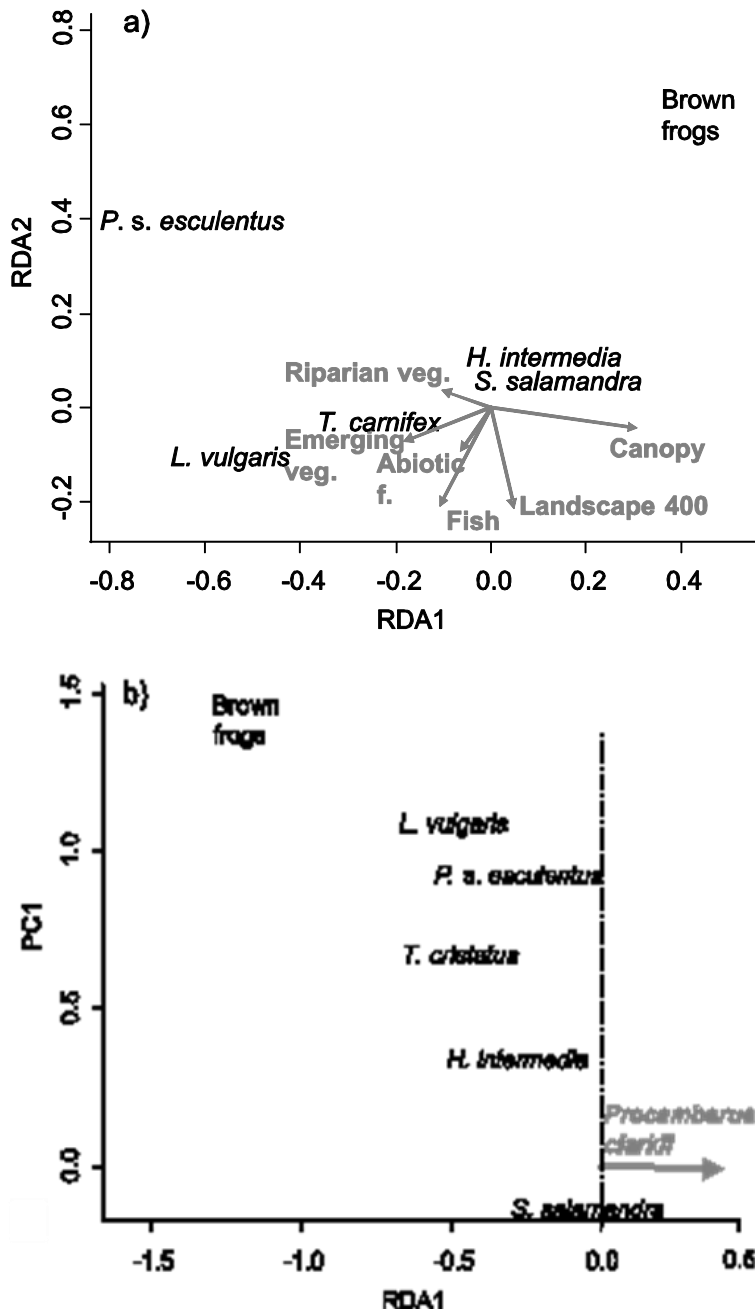
Figure 3. Results of constrained redundancy analysis showing (a) the relationship between wetland features and the abundance of amphibian larvae while taking into account spatial autocorrelation; (b) the relationship between the abundance of amphibian larvae, and the abundance of *Procambarus clarkii*, while taking into account wetland features and autocorrelation. Constraining variables are in grey colour.

Table 4. Models relating the abundance of amphibian larvae to environmental features and abundance of *P. clarkii*: comparison of models with and without the effect of *P. clarkii*. Significance values refer to the effect of *P. clarkii*; SEVM was used for *L. vulgaris* only, because of the spatial autocorrelation of residuals. See Table 2 for abbreviations.

Species	SEVM	Environmental only				Environmental + *P. clarkii*						
		K	R^2	AIC	w	K	R^2	AIC	w	F	d.f.	P
L. vulgaris	Y	4	0.52	132.2	0.08	5	0.61	127.4	0.92	6.4	1,29	0.017
T. carnifex	N	1	-	123.9	<0.01	2	0.36	110.6	0.99	24.6	1,32	0.0002
H. intermedia	N	3	0.03	132.3	0.16	4	0.17	129.0	0.84	5.0	1,31	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
P. s. *esculentus*	N	3	0.02	133.7	0.07	4	0.20	138.9	0.93	7.1	1,31	0.012

4.4. DISCUSSION

We observed strong relationships between the invasive crayfish *P. clarkii* and amphibian communities, but the estimates of its influence, based on species distribution data, were different from those obtained through analysis of the abundance of larvae (Figures 1b, and 3). 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 related to *P. clarkii* (i.e., did not breed in invaded wetlands), while others (pool frog and brown frogs) were apparently associated with invaded wetlands (Tables 2 and 3, Figure 1b). Observing such a result, without considering data on breeding success, would suggest the misleading 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 related to the distribution of the alien crayfish, suggesting a strong impact on reproductive success (Table 4, Figures 2 and 3). Therefore, the alien crayfish may affect amphibian communities through multiple mechanisms: loss of suitable breeding sites, and loss of fitness in sites 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., 2006a). 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 (Griffiths, 1995). Therefore, adults may directly experience predation by crayfish and avoid invaded wetlands. Native species may also avoid invaded sites because AIS alter environmental features and make them unsuitable (Didham et al., 2007). *Procambarus 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 (Cruz et al., 2006b; Hartel et al., 2010; Strijbosch, 1979). In these cases, *P. clarkii* may negatively impact amphibians through the loss of breeding wetlands. In human dominated landscapes, the number of suitable breeding wetlands is already limited, and loss of

breeding sites may quickly disrupt metapopulation dynamics and determine local extinction (Ficetola and 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 preyed. Experiments demonstrated that *P. clarkii* is able to prey on larvae of most European amphibians (Cruz and Rebelo, 2005; Gherardi et al., 2001); crayfish predation is therefore the most likely cause of the negative relationship between crayfish and tadpole abundance (Figure 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 (Figure 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 detected, 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. Amphibians are able to detect the presence of some predators in wetlands, and avoid breeding in these environments (Resetarits Jr, 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.

Our measure of larval abundance can not be used as a proxy of larval survival, because absence of larvae may indicate either low reproductive effort, or high larval mortality. An accurate estimate of fitness would require an exhaustive measurement of the number 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 (Dodd, 2010; Karraker and Gibbs, 2009), 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. Therefore, we believe that information obtained through larval sampling is useful to compare breeding performance among ponds.

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 AIS 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 hydroperiod allows a prolonged activity period, while reproductive success can be limited in ephemeral wetlands (Cruz and Rebelo, 2007; Gutierréz-Yurrita and Montes, 1999) see also the chapter 2 of this thesis. 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) where similar to the ones detected by previous studies in uninvaded areas (e.g., Denoël and Ficetola, 2008; Ficetola and De Bernardi, 2004; Indermaur et al., 2010; Pavignano et al., 1990). In practice, most species were associated with relatively large and permanent wetlands, contrary to the predictions of the habitat use change hypothesis (Figure 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 (Ficetola et al., 2011a; Skelly and 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 AIS.

AIS are a major cause of the decline of biodiversity but, when a new species invades a biota, the consequences can be not evident for long periods (Strayer et al., 2006). Early understanding of the impact over native species may allow more prompt management actions, with increased possibility of success. In our study case, considering the

distribution of adult amphibians only would lead to misleading conclusions, as adult amphibians 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 AIS will have in the next future. These information may allow managers to start conservation actions before than consequences of AIS 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 and Panov, 2009).
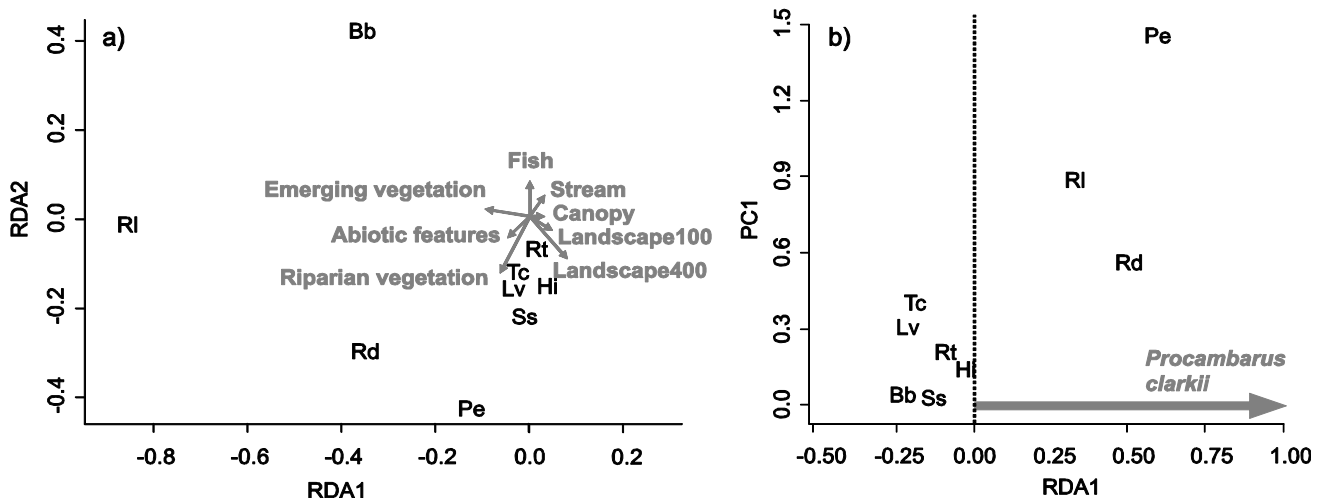
Supporting information

Figure S1. 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 distributing and presence of *Procambarus clarkii*, while taking into account wetland features and autocorrelation. For *T. carnifex*, *H. intermedia* and *P.* s. *esculentus* species occupancy was estimated using PRESENCE. Species codes are described in Figure 2; constraining variables are in grey colour.

Table S1. Relationships between species distribution, environmental features and presenceof *P. clarkii*: models using probability of presence, as estimated by occupancy models. R^2_N: Nagelkerke's R^2; K: number of parameters in the model; AIC: Akaike's Information Criterion; w: AIC weight.

Hyla intermedia		Best AIC models								Effect of *P. clakii*		
	sign	*F*	df	*P*	R^2	K	AIC	w		*F*	df	*P*
a abiotic features	+	4.1	1,121	0.045	0.22	3	90.1	0.54		0.3	1,120	0.578
landscape 400	+	4.2	1,121	0.043								
SEVM eigv.		29.1	1,121	<0.001								
b canopy cover	-	6.0	1,122	0.016	0.20	2	90.4	0.46		1.1	1,121	0.301
SEVM eigv.		23.4	1,122	<0.001								

Pelophylax s. *esculentus*		Model including *P. clarkii*								Model without *P. clarkii*		
										K	AIC	w
P. clarki	+	8.5	1,122	0.029	0.11	2	218.9	0.955		1	225.0	0.045
landscape 400	+	10.9	1,122	0.004								

5.

Impact of an invasive crayfish on species with complex life cycles: a case study with odonates

ABSTRACT

The temporal dimension is a key parameter when analysing the impact of alien invasive species. Studies on early invasion stages allow to better understand how ongoing processes modify native communities helping to plan effective management actions. *Procambarus clarkii* is an invasive crayfish that alters multiple features of invaded wetlands but its impact on organisms with complex life cycles is little investigated. We considered 121 wetlands, and we used both quantitative and qualitative data to evaluate the relationships between *P. clarkii* and the richness of adults, larvae, and exuviae of odonates. We recorded environmental features of each wetland and we used GIS to measure the natural vegetation in the surrounding landscape. We performed analysis using spatially explicit techniques (Generalized Linear Models and spatial Generalized Additive Models) allowing the integration of spatial autocorrelation into analyses. Spatial autocorrelation, wetland features and landscape features explained a significant amount of odonate community richness. We observed a significant relationship between *P. clarkii* and the odonate community, but the effect of the invasive crayfish on the three odonate stages was different: species richness measured using both larvae and exuviae was negatively related to the crayfish presence, while negative effects on adults were less evident. At early stages of the invasion, larvae and exuviae may be more helpful for the assessment of the impact of invasive species, while adults may better describe the long term consequences of an invasion at the landscape scale. The invasion of *P. clarkii* has different negative impact on different stages of species with complex life cycles.

KEY WORDS: *Procambarus clarkii*, biological invasions, freshwater ecosystems, early assessment, environmental features, differential impact mechanisms.

5.1. INTRODUCTION

There are approximately 50.000 alien invasive species in the United States and 11.000 are inventoried in Europe (Drake, 2009; Pimentel et al., 2005); alien invasive species (AIS) have been recognized as one of the major causes of biodiversity loss. Many native ecosystems have been irretrievably modified by AIS, determining the decline and even the extinction of thousands of native species (Drake, 2009; Gherardi, 2007; Wittenberg and Cock, 2001).

AIS can damage native species through multiple mechanisms, including competition, predation, spread of diseases and hybridization (Pimentel, 2002). Prevention of new introductions is the optimal strategy to avoid the negative consequences of AIS, but If this is not feasible, earlier management actions, at the same cost, have the greater effectiveness (Allendorf and Lundquist, 2003; Polasky, 2010; Wittenberg and Cock, 2001).

Only a subset of alien species become invasive, showing clear negative impact on native ecosystems (Jeschke and Strayer, 2005; Suarez et al., 2005; Vilà et al., 2010), and priority is given to manage species that clearly show negative effects on native habitats. Research is therefore required to assess environmental impact of alien species and help setting correct management actions (Vilà et al., 2010; Wittenberg and Cock, 2001).

Inland waters are particularly exposed to alien species invasions: hundreds of freshwater species have been moved outside of their native ranges as a direct consequence of the use of this ecosystem for human activities (Gherardi et al., 2008; Strayer, 2010), and the deterioration of freshwater habitats is determining species extinction at rates even five times higher than those for terrestrial habitats (Ricciardi and Rasmussen, 1999).

AIS can strongly stress the invaded environments, being part of a complex web of environmental interactions that involves environmental stressors, alien species, and native communities, furthermore, AIS introductions are more frequent where human activities alter the environment and assist their spread (Didham et al., 2007). Analyses considering both habitat modifications and AIS are therefore needed to take apart their relative role (Didham et al., 2007; Strayer, 2010). Nevertheless, only a small subset of studies considered

the effects of AIS concurrently to the ones of environmental modifications, such as urbanization or the degradation of natural environments, that can have negative non-additive interactions with native species.

Biological invasions can be described as multi-step processes: first, the introduced species must arrive, survive, and establish itself in a new area; second, the alien species develops an invasive behavior, spreads and impacts on native environment and native species (Allendorf and Lundquist, 2003). Over time, during the different invasion stages, in invaded habitats occur complex ecological and evolutionary processes, such as changes in the invaded biological community, changes in the species that invade, changes in the abiotic environment, and changes in the interaction between the ecosystem components; the temporal dimension is therefore a key parameter that needs to be considered when analysing the effects of AIS (Strayer et al., 2006).

Research on later phases of invasion often describes widely altered communities, in contrast, the study of early stages, when the AIS has not completed the habitat invasion, allow to better understand how ongoing processes modify the invaded communities. Research on early invasion stages may also helps to plan more effective and less costly management actions, helping to prevent further damages and AIS spread (Polasky, 2010).

The red swamp crayfish *Procambarus clarkii* is a freshwater AIS native of Eastern North America, now invasive in five continents (Barbaresi and Gherardi 2000; Huner 1994); it is considered one of the 100 worst AIS in Europe (Drake, 2009); it has high fecundity and great ecological plasticity that determine its high invasiveness (Aquiloni and Gherardi, 2008; Gherardi, 2006; Hazlett et al., 2003; Huner and Lindqvist, 1991). *Procambarus clarkii* is a polytrophic opportunistic crayfish that can change the habitat features, altering the flow of nutrients, and having multiple negative consequences on native species and ecosystems (Gherardi, 2006; Matsuzaki et al., 2009). *Procambarus clarkii* can feed on detritus, macrophytes, molluscs, fishes, amphibians and insects (Gherardi, 2006; Souty-Grosset et al., 2006), altering the biodiversity of macrobenthic and odonate communities (McCarthy et al., 2006; Nyström et al., 1996; Wilson et al., 2004).

Odonates are often used in ecological studies as surrogates of wetland biodiversity and to assess water-body health (Oertli, 2008; Sahlen and Ekestubbe, 2001; Samways, 2008), and among insects, odonates are one of the orders to which the greater conservation attention is devoted; for instance, they are the first insect order for which researchers attempted a global evaluation of the conservation status using the IUCN Red List Categories (Clausnitzer et al., 2009). Previous researches gave clear indications on the multiple negative effects of *P. clarkii* invasion on native ecosystems. Laboratory tests and gut contents analysis showed that *P. clarkii* can feed on odonates, even preferring them to other food like living fish, and vegetal matter (Correia, 2003; Ilhéu and Bernardo, 1993). However, information on the impact of *P. clarkii* on odonate communities in natural environments remains scarce.

This study evaluates the relationships between *P. clarkii* and the richness of odonate communities in an area recently invaded by this crayfish. Odonates show a complex life cycle (Wilbur, 1980), with aquatic larvae and adults living in sub-aerial conditions. AIS can therefore have a different impact on the different life cycle stages (Corbet, 2004; Córdoba-Aguilar, 2008). For instance: (1) AIS can determine the complete disappearance of odonates in the invaded areas; (2) adult odonates may be present in the invaded areas, but avoid breeding in invaded wetlands; (3) adult odonates could attempt breeding in invaded wetlands, but the AIS hamper breeding success.

Analysing relationships between AIS and the different life cycle stages can help to disentangle the mechanisms determining the AIS impact on odonates (McCauley et al., 2008; Serrano-Meneses et al., 2008). In particular, we evaluated the relationships between *P. clarkii* and (1) the richness of adults; (2) the richness and abundance of larvae, and (3) the richness of exuviae. Adults, showing the greater dispersal ability, determine the distribution of dragonfly communities; Larvae indicate that odonates grow in wetlands, and exuviae indicate the successful larval development and the complement of the life cycle (Corbet, 2004; Córdoba-Aguilar, 2008). In all our analyses we also considered the

spatial autocorrelation and environmental features to take into account their potential effects.

5.2. METHODS

Study area and methods outline

We considered 121 waterbodies (ponds, slow streams, ditches, and small lakes) in Lombardy region, Northern Italy, in an area including the upper Po River plain and the foothills of Brianza, at altitudes ranging between 132 m and 397 m; annual average temperature between 10 °C and 14.4 °C, and annual mean rainfall between 1400 mm and 1600 mm (Gerletti and Marchetti 1977). The landscape of this region is human dominated (Figure 1), with large agricultural and urban areas, and is characterized by a complex network of lakes, ponds, rivers, and canals. In this region, several natural parks host important freshwater communities. The area was recently invaded by *P. clarkii,* approximately between the years 2004 and 2006 (Fea et al., 2006), and *P. clarkii* currently has an highly fragmented distribution in the area (chapter 2). We surveyed 108 wetlands during 2009 and 13 further wetlands during 2010 (total 121).

We performed 6 surveys for each wetland, searching for odonate adults, exuviae ,and larvae, and for *P. clarkii*; in a subset wetlands we performed an additional survey sampling quantitatively both odonate larvae and *P. clarkii*; we described each sampled wetland using a standard set of environmental parameters; taking into account the environmental parameters, surveys were performed to assess relationships between odonate distribution and *P. clarkii*, and to derive guidance on the reproductive success and reciprocal influence.

The spatial autocorrelation is a phenomenon affecting the distribution of both native populations and exotic species (Hamasaki et al., 2009; Smolik et al., 2010), resulting in a higher similarity of species assemblages occupying closer localities. Species

distribution is, in fact, limited by endogenous factors due to species ecology and by exogenous factors that comprises autocorrelated environmental features (e.g., vegetation, climate, and landscape composition) (Beale et al., 2010; Dormann, 2007), therefore we integrated spatial autocorrelation into all our analyses.
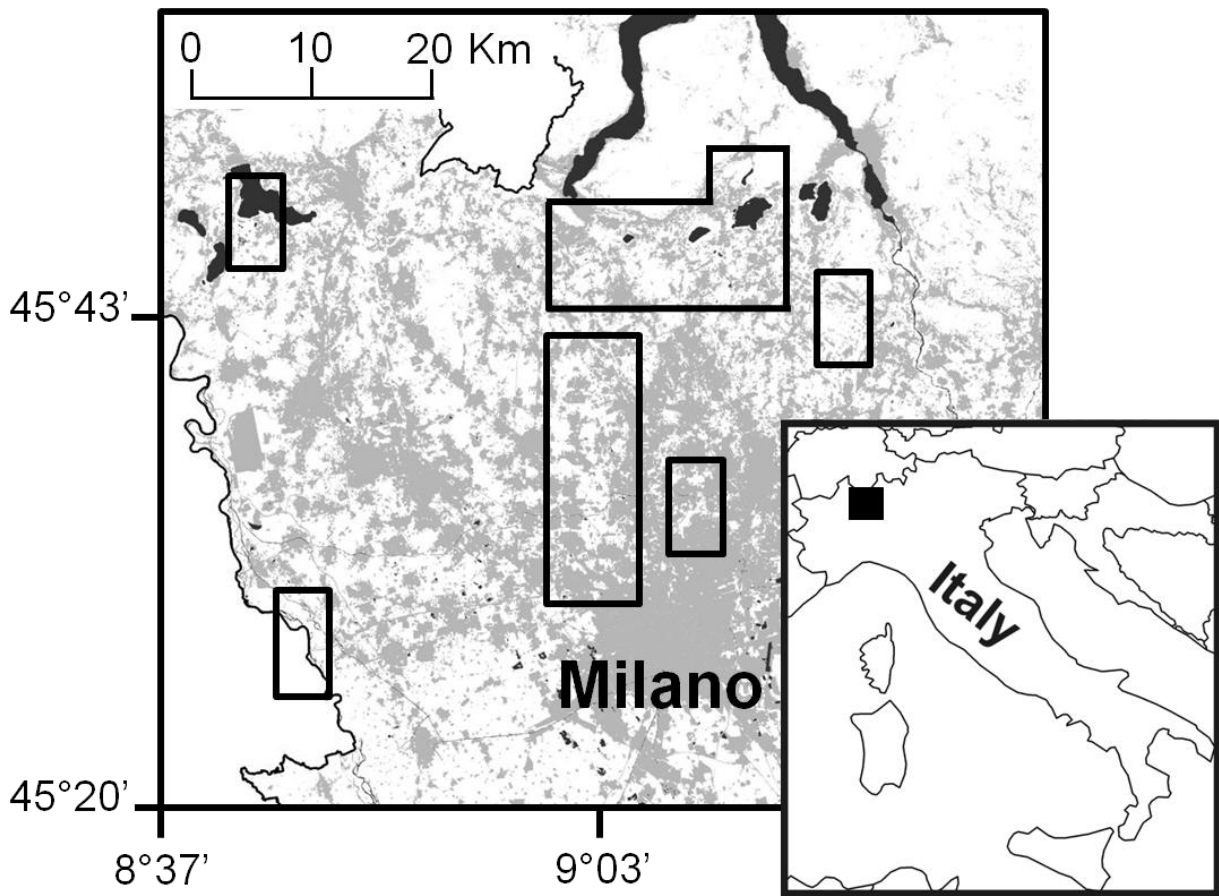

Figure 1. Study area (Lombardy region, Northern Italy). Black lines enclose the areas where wetlands are located, dark shaded areas represent lakes and major rivers, light shaded areas represent urban areas.

Odonate communities and *P. clarkii* surveys

From May to August 2009, we used a combination of multiple techniques to assess the presence of odonates and of *P. clarkii*. We performed at least 4 surveys in daytime and two surveys after dusk.

For *P. clarkii*, we used nocturnal and diurnal visual census of individuals and exuviae helped by dip netting (Reynolds et al., 2006).

For odonates, we explored wetlands during the central hours of the day, with sunny weather and little wind speed or no wind; during each survey we looked for adults, last instar exuviae, and larvae using visual census helped by aerial net, binoculars, and magnifying lens for adults, and deep net for larvae. A sweep net helped exuviae detection, especially in temporary wetlands where, due to the broad water level fluctuations, banks have a wide and indeterminate extension. Adults are the less demanding odonate life stage to study but surveys including the three studied stages give more detailed information. Exuviae, in particular, give useful indications on all other life stages and denote the reproductive success at breeding sites (Córdoba-Aguilar, 2008; Ott, 2010).

Quantitative sampling of larval odonates and *P. clarkii*

In late May-early June 2010 we performed an additional survey using pipe sampling to asses quantitatively the abundance of odonate larvae (McCauley et al., 2008; Werner et al., 2009).

Pipe sampling was focused on a subsample of 34 wetlands keeping a balanced ratio (50%) between wetlands invaded and not invaded by the invasive crayfish.

We used a 0.25 m² circular pipe sampler; samples were collected by quietly approaching an area and quickly sinking the pipe through the water column into the sediments, to seal the sample area. We used small nets (1 mm mesh size) to remove all animals from the water column and from the first centimeters of the sediment. Net sweeps

were stopped after at least 10 consecutive performed sweeps without capturing any animal (Werner et al., 2009). We collected a number of samples proportional to wetland surface (average samples number: four).

Odonate larvae were identified following standard keys (Carchini, 1983; Heidemann and Seidenbusch, 2002). Because of the morphological overlapping, particularly wide during early larval stages, in our analysis we pooled larvae of the following species as singles species groups: (1) *Coenagrion puella* and *C. pulchellum* as *C. puella-pulchellum;* (2) *Sympetrum sanguineum* and *S. striolatum* as *S. sanguineum-striolatum.* All Individuals of *P. clarkii* were weighed to measure their mass (g / m²).

Environmental variables

In late May-June, we recorded eight parameters used as standard characterization of wetlands: surface area, maximum depth, water permanence, average stream velocity, presence of fishes, canopy cover, percentage of riparian vegetation along the shoreline, percentage of emerging or floating vegetation on wetland surface (Table 1). Parameters were recorded using standard bio-assessment protocols; vegetation cover was visually estimated (Table 1), details on their measurement are reported elsewhere (Barbour *et al.*, 1999; Van Buskirk, 2005; Ficetola *et al.*, 2011b). Furthermore, as odonate communities are affected by landscape features (Hamasaki et al., 2009; Samways and Steytler, 1996), we used the ArcView GIS (© ESRI, 1999) to measure the percentage of natural vegetation in the landscape surrounding each wetland, on the basis of the 2008 vector map of the Lombardy region (www.cartografia.regione.lombardia.it). Natural vegetation was measured in a radius of 400 m from each wetland.

Table 1. Environmental variables recorded.

Environmental variables
a: wetland
Surface[1] (m)
Maximum depth[1] (cm)
Permanence during the study period (Y/N)
Stream velocity[1] (m/s)
Fish presence (Y/N, visual estimate)
Canopy cover (%, visual estimate)[2]
Emerging vegetation (% of vegetation emerging from water surface,visual estimate)[2]
Riparian vegetation (%,visual estimate)[2]
b: landscape
Natural vegetation (%) [2]

1: Log-transformed prior to analyse.

2: square-root arcsin transformed prior to analyse.

Statistical analyses

We used Generalized linear models (GLM) and generalized additive models (GAM) to assess the relationships between the richness of odonate communities, the presence of *P. clarkii*, and the environmental features. We repeated analyses three times (adults, larvae, and exuviae) and at three levels (overall community, Anisoptera only, and Zygoptera only). In all analysis, community richness (number of species) was the dependent variable, while we included environmental variables and *P. clarkii* as dependent variables.

For the analysis of adults and exuviae, we considered 107 wetlands and the distribution of *P. clarkii* were described by presence-absence data. For the analysis of larvae, we considered 34 wetlands, and, in this case, the distribution of *P. clarkii* were described by its abundance (g / m^2).

In the analysis of adults and exuviae, residuals of preliminary GLMs where spatially autocorrelated. Spatial autocorrelation may bias the results of standard regression techniques (Beale et al., 2010), therefore we used spatial GAM to assess the relationships between community richness, presence of *P. clarkii* and environmental features, while taking into account the effect of spatial autocorrelation. Spatial GAMs incorporate the coordinates of localities in GAMs as covariates (Beale et al., 2010). Simulations showed that spatial GAMs are among the techniques with lowest bias, and with good performance, even in presence of violations of assumptions, such as nonstationarity of spatial autocorrelation (Beale et al., 2010).

For the analysis of Zygoptera exuviae, residual deviance was much lower than one (variance inflation factor = 0.39), therefore we used a quasi-Poisson family instead then using a Poisson family, and we calculated significance using an *F* test instead than using an χ^2 test (Crawley, 2007). To confirm that our results were not affected by the statistical approach, we also performed preliminary analyses using Spatial Eigenvector Mapping (Dormann et al., 2007); these analyses yielded the same results.

For the analysis of larval community richness, we had a limited number of wetlands (N = 34); including too many environmental variables in a model would lead to the risk of overfitting models; therefore, we used Akaike's Information Criterion (AIC) to identify the minimum adequate model (MAM) explaining community richness on the basis of a limited number of independent variables. We built GLMs (Poisson error distribution), corresponding to all combinations of environmental variables, and we calculated the AIC for each model. We identified the model with lowest AIC as the MAM, which can be defined as the model explaining most variance with the lowest number of variables (Diniz-Filho et al., 2008). For this analysis, estimates of the abundance of *P. clarkii* (g / m²) where available, therefore we used as independent variable crayfish abundance instead than crayfish presence / absence. For the analysis of larval communities, residuals were not spatially autocorrelated (permutation test, $P > 0.05$ for all models) (Lichstein et al., 2002). In all analyses, environmental variables were not strongly correlated among them (for all pairwise correlations, $|r| < 0.4$). We performed analyses in the R statistical environment (www.r-project.org).

5.3. RESULTS

We detected *P. clarkii* in 33 wetlands (31%). During our surveys we detected the presence of forty-two odonates species, 17 Zygoptera and 25 Anisoptera (Tables 2 and 3). The average richness was 6.6 species per wetland (SD = 4.1).

Adult communities richness

If data of adults are considered, the richest odonate communities were associated to relatively deep, permanent, and standing waters, with abundant emerging vegetation, and within landscapes with natural vegetation. The relationship between community richness and presence of *P. clarkii* was not significant (Table 4a).

Relationships between community richness and environmental variables remained the same if only Anisoptera are considered (Table 4b).

The situation was different for Zygoptera. Data from adults suggest that the richest communities are associated to permanent wetlands with presence of *P. clarkii* (Table 4c). For all analyses, spatial autocorrelation explained a significant amount of variation.

Larval community richness

We detected *P. clarkii* in 50% of wetlands surveyed with pipe sampling; furthermore, we detected larvae of fourteen odonate taxa (Tables 2 and 3); in invaded wetlands we found seven odonate taxa: *Lestes viridis, Ischnura elegans, C. puella-pulchellum, Aeshna cyanea, Orthetrum albistylum, Orthetrum cancellatum,* and *S. sanguineum-striolatum.*

The overall richness of odonate larval communities was negatively related to the abundance of *P. clarkii*. Furthermore, we observed the richest larval communities in deep wetlands within the most natural landscapes (Table 5a). The relationship between community richness and *P. clarkii* was negative and significant also if Anisoptera are analysed separately (Table 5b).

For Zygoptera, the richest larval communities were associated to the wetlands with lower canopy cover (i.e., sunny wetlands) (Table 5c). The abundance of *P. clarkii* was not included in the MAM of Zygoptera. The relationship between abundance of *P. clarkii* and richness of Zygoptera was not significant both if *P. clarkii* was added to the MAM (χ^2_1 = 0.1, *P* = 0.829) and if *P. clarkii* was included as unique predictor of Zygoptera richness (χ^2_1 = 0.9, *P* = 0.349).

Exuviae richness

We detected exuviae of eighteen odonate taxa (Tables 2 and 3); in invaded wetlands we found eight species of odonates: *Calopteryx virgo, C. splendens, Lestes viridis, Platycnemis*

pennipes, *Coenagrion puella*, *Aeshna isoceles*, *Orthetrum cancellatum*, and *Sympetrum sanguineum*.

If exuviae are considered, the richness of odonate communities was negatively related to the abundance of *P. clarkii* (Table 6a); the relationship between community richness and *P. clarkii* was negative and significant also if Anisoptera and Zygoptera are analysed separately (Tables 6b and 6c).

Relationships between community richness and environmental variables were weak; the only significant relationship was a lower richness of Zygoptera in running waters. For all analyses, spatial autocorrelation explained a significant amount of variation.

Table 2. Odonates detected: Zygoptera species, relative adults frequency, and indications on the species detection as exuvia and larva.

Species found	Adult frequency	Larva detected	Exuvia detected
Suborder Zygoptera			
Calopteryx splendens	0.18	*	*
Calopteryx virgo	0.28		*
Lestes barbarus	0.01		
Lestes dryas	0	*	
Lestes sponsa	0.03		
Lestes virens	0.02	*	
Lestes viridis	0.16	*	*
Platycnemis pennipes	0.20	*	*
Pyrrhosoma nymphula	0.06		
Ischnura elegans	0.53	*	*
Ischnura pumilio	0.07		*
Erythromma viridulum	0.06		
Erythromma lindenii	0.07		*
Enallagma cyathigerum	0.03		
Coenagrion puella	0.43	*1	*
Coenagrion pulchellum	0.14	*1	
Ceriagrion tenellum	0.17		

*: species detected.

1: species merged in a single taxon for analyses.

Table 3. Odonates detected: Anisoptera species, relative adults frequency, and indications on the species detection as exuvia and larva.

Species found	Adult frequency	Larva detected	Exuvia detected
Suborder Anisoptera			
Aeshna affinis	0.06		
Aeshna cyanea	0.21	*	*
Aeshna isoceles	0.36	*	*
Aeshna mixta	0.04	*	
Anax imperator	0.39		*
Anax parthenope	0.27		*
Onychogomphus forcipatus	0.04		
Onychogomphus uncatus	0.02		
Cordulegaster bidentata	0.04		
Cordulegaster boltonii	0.13		*
Cordulia aenea	0.03		
Somatochlora flavomaculata	0.26		
Somatochlora metallica	0.02		
Libellula depressa	0.15		
Libellula fulva	0.16		*
Libellula quadrimaculata	0.12	*	*
Orthetrum albistylum	0.30	*	
Orthetrum brunneum	0.01		
Orthetrum cancellatum	0.30	*	*
Orthetrum coerulescens	0.21		
Crocothemis erythraea	0.21		
Sympetrum fonscolombii	0.11		*
Sympetrum pedemontanum	0.01		
Sympetrum sanguineum	0.62	***1**	*
Sympetrum striolatum	0.04	***1**	

*: species detected.

 1: species merged in a single taxon for analyses.

Table 4. Relationships between the richness of odonate adult communities, the presence of *P. clarkii* ,and environmental features, as explained by generalized additive models (GAM).

Analyses level	Variables	*B*	*χ*	df	*P*
a. Overall community					
	P. clarkii	0.12	1.1	1	0.297
	stream	-2.69	9.0	1	0.003
	Area	0.07	0.4	1	0.529
	depth	0.43	7.3	1	0.007
	canopy	-0.17	2.8	1	0.096
	riparian veg.	0.00	0.0	1	0.974
	emerging veg.	0.25	5.2	1	0.022
	permanence	1.17	30.0	1	<0.001
	landscape veg.	0.54	5.8	1	0.016
	Spatial variables		52.2	10	<0.001
b. Anisoptera only					
	P. clarkii	-0.02	0.0		0.908
	stream	-3.02	6.1		0.014
	area	0.03	0.1		0.801
	depth	0.42	4.6		0.031
	canopy	-0.23	3.3		0.068
	riparian veg.	-0.06	0.2		0.655
	emerging veg.	0.27	4.0		0.047
	permanence	0.94	15.9		<0.001
	landscape veg.	0.58	4.2		0.040
	Spatial variables		34.6	10	<0.001
c. Zygoptera only					
	P. clarkii	1.06	19.7		<0.001
	stream	-1.48	1.7		0.197
	area	0.13	0.7		0.419
	depth	0.29	1.3		0.251
	canopy	-0.04	0.1		0.810
	riparian veg.	0.08	0.3		0.609
	emerging veg.	0.17	1.0		0.325
	permanence	2.07	15.4		<0.001
	landscape veg.	0.49	2.0		0.157
	Spatial variables		15.7	8	0.038

Table 5. Relationships between the richness of odonate larval communities, the presence of *P. clarkii*, and environmental features, as explained by the minimum adequate model (MAM) identified using Akaike's Information Criterion (AIC) among Poisson error distribution GLMs of all combinations of environmental variables.

Analyses level	Variables	*B*	*χ*	df	*P*
a. Overall community					
	P. clarkii	-0.31	9.2	1	0.002
	depth	0.863	3.9	1	0.049
	landscape veg.	1.63	4.2	1	0.041
b. Anisoptera only					
	P. clarkii	-0.29	5.4		0.021
c. Zygoptera only					
	canopy	-1.38	5.1	1	0.025

Table 6. Relationships between the richness of odonate exuviae, the presence of *P. clarkii*, and environmental features, as explained by generalized additive models (GAM).

Analyses level	Variables	*B*	X	df	*P*
a. Overall community					
	P. clarkii	-1.20	6.0	1	0.014
	stream	-3.81	0.9	1	0.347
	area	-0.10	0.1	1	0.752
	depth	0.46	0.9	1	0.356
	canopy	-0.16	0.2	1	0.649
	riparian veg.	0.00	<0.1	1	0.992
	emerging veg.	-0.04	<0.1	1	0.932
	permanence	153.30	<0.1	1	0.999
	landscape veg.	0.05	<0.1	1	0.944
	Spatial variables		23.3	7	0.001
b. Anisoptera only					
	P. clarkii	-1.67	6.9		0.009
	stream	-3.88	0.5		0.482
	area	-0.04	0.0		0.919
	depth	0.70	1.2		0.265
	canopy	-0.16	0.1		0.722
	riparian veg.	-0.23	0.2		0.636
	emerging veg.	0.36	0.5		0.500
	permanence	140.80	<0.1		0.999
	landscape veg.	0.35	0.1		0.709
	Spatial variables		16.1	3	0.001
c. Zygoptera only					
	P. clarkii	-1.95	6.4	1,86	0.014
	stream	-21.83	9.1	1,86	0.003
	area	0.42	0.8	1,86	0.361
	depth	2.23	7.4	1,86	0.008
	canopy	0.46	1.2	1,86	0.274
	riparian veg.	0.41	0.7	1,86	0.395
	emerging veg.	0.41	0.7	1,86	0.409
	permanence	134.30	<0.1	1,86	0.999
	landscape veg.	0.49	0.2	1,86	0.634
	Spatial variables		2.8	12,86	0.003

5.4. DISCUSSION

The number of taxa found in the study area (Tables 2 and 3) accounts for 62% of species of the total regional fauna (Balestrazzi and Pavesi, 2008), suggesting that our sampling protocol exhaustively covered the odonate biodiversity of the study area.

We observed a strong relationship between *P. clarkii* and the odonate communities, but the effect of the invasive crayfish on the native species distribution was different when considering separately the distribution of odonate adults, larvae, and exuviae. In our analyses features of wetlands and of the surrounding landscape where the main drivers of adult community richness. Adult richness was higher in relatively deep, permanent, and standing waters, with abundant emerging vegetation, within landscapes with natural vegetation. The analysis of Anisoptera richness was in agreement with the analysis of all odonates. Conversely, if analysed separately, adult Zygoptera showed only a positive relationship with permanent wetlands (Table 4). Our results are similar to those reported for others regions (Mabry and Dettman, 2010; McCauley et al., 2008; Sahlen and Ekestubbe, 2001), confirming the importance of these environmental variables for odonate communities.

The richness of adult community was not negatively related to the presence of the invasive crayfish, actually, if Zygoptera are analysed separately, our results suggest a positive relationship between adult damseflies richness and the invasive crayfish (Table 4). Limiting our analysis to adult distribution would lead to the incorrect conclusion that the invasive crayfish has not an adverse effect on odonate communities, and might even have a positive effect. Performing a separate analysis on different odonate life history stages helps to draw out a more detailed description of the actual impact of crayfish invasion on odonates.

When larvae are analysed (Table 5), we found the association of richest communities with relatively deep wetlands, in landscapes with natural vegetation; these relationship remained positive if Anisoptera are considered separately, while for

Zygoptera we found a positive relationship only with the more sunny wetlands. Larval community richness was negatively related to the abundance of the invasive crayfish both if the overall community was considered, and if Anisoptera were analysed separately. Conversely, the richness of Zygoptera larvae was not related to the crayfish abundance, even if adults were associated with *P. clarkii.*

Exuviae give the best indication of the successful odonate reproduction within a wetland (Oertli, 2008), and all the analyses of exuviae confirmed the negative relationship between the invasive crayfish and the odonate community richness (Table 6), this negative relationship was strong also for Zygoptera, even if analyses suggested different patterns in adults and larvae (Tables 4c, 5c, and 6c).

Our analysis shows the multiple steps of the dramatic impact of the crayfish invasion on odonate communities. In early invaded wetlands *P. clarkii* can have the strongest impact on juvenile aquatic stages of odonates; this negative effect could be the result of both direct and indirect interactions: *P. clarkii* can directly prey on larval odonates reducing their number (Correia, 2003; Ilhéu and Bernardo, 1993; Maezono and Miyashita, 2004; McCarthy et al., 2006; Nyström et al., 1996), but it can also negatively affect richness and abundance of the macrobenthic community, reducing invertebrates and amphibian larvae that constitute the main prey of larval odonates (Correia and Anastacio, 2008; Cruz et al., 2008). Furthermore aquatic predators, such as *P. clarkii*, can induce defensive mechanisms on dragonflies that negatively affect their feeding activity and metabolism rate (Heads, 1985; Serrano-Meneses et al., 2008). Processes that slow the larval growth, forcing larval odonates to remain longer in water, expose them for longer to mortality sources like predation and habitat drying; furthermore, adults can emerge late, and at smaller size, with potential negative effects on their fecundity (McPeek, 2008; Serrano-Meneses et al., 2008; Stoks et al., 2008).

Odonates are strongly associated with macrophytes: during larval stages they use the aquatic vegetation for anchorage, concealment, defence, and as foraging sites (Corbet, 2004; Córdoba-Aguilar, 2008). The destruction of macrophytes by the invasive crayfish

(Gherardi and Acquistapace, 2007) can negatively impact larval stages, in fact, by consuming plant material, *P. clarkii* could also destroy eggs laid on this substrate, with further negative consequences on abundance of larval population.

Exuviae analysis confirmed that the invasive crayfish leads to a generalized environmental deterioration (Gherardi, 2006; Gherardi and Panov, 2009) making the aquatic environment unsuitable for the development of juvenile stages.

Procambarus clarkii could therefore negatively affect odonate communities through two, non-exclusive, main mechanisms: loss of breeding sites, and fall of the reproductive success at the breeding sites.

Adults of some odonate species could avoid reproduction in wetlands where *P. clarkii* is present as a consequence of chemical or mechanical signals directly related to the presence of the crayfish, or because of the environmental changes caused by its presence; for instance, *P. clarkii* removes aquatic macrophytes, which are important factors for the selection of breeding sites for odonates (Corbet, 2004; Córdoba-Aguilar, 2008; Maezono and Miyashita, 2004; Sahlen and Ekestubbe, 2001). The analysis of adults does not support this hypothesis because, after taking into account environmental features, we found no negative relationships between the presence of *P. clarkii* and the presence of adult odonates; furthermore, previous studies suggest that, for the selection of reproduction sites, odonates use mainly visual and tactile cues based on habitat structure, while they have a limited ability to detect predators (Corbet, 2004; Crumrine et al., 2008). Nevertheless, high density populations of *P. clarkii* could directly negatively affect deposition of endophytic species, by attempting the predation of adults that, during eggs laying, spend long time submerged in the water.

Adults of other odonate species breed in the invaded wetlands; this strategy, that in our data is evident for Zygoptera, might arise because of lack of detection of cues, or by the reduction of odonate competitors and predators like fishes, insects, and larger dragonflies (Blaustein et al., 2004; Crumrine, 2010; Gydemo et al., 1990; Pierce et al., 1985).

However the negative relationship with *P. clarkii* observed with the exuviae analysis indicate that this strategy would lead to the fall of the odonate reproductive success.

Spatial autocorrelation explained a significant component of community richness (Tables 4 and 6), this probably arises because dispersal is extremely important to maintain odonate communities; sites neighbouring species-rich wetlands therefore host more species than expected on the basis of the sole environmental features (Hamasaki et al., 2009; McCauley et al., 2008; Yamanaka et al., 2009). Within the study area, only a subset of wetlands are invaded by *P. clarkii*, therefore non invaded sites could support natural populations that disperse in the surrounding landscape and attempt reproduction in invaded wetlands. Unfortunately, simulation and empirical studies showed that, if the proportion of suitable patches in a given landscape falls below a given threshold, dispersal can not anymore maintain populations, and species can undergo abrupt extinction (Fahrig, 2002; Ficetola and Denoël, 2009). The community of odonates can be therefore maintained only if the numbers of wetlands without *P. clarkii*, where odonates can attain positive fitness, remain high.

Procambarus clarkii is a generalist crayfish that is well adapted to live in temporary wetlands with short hydroperiod, even shorter than 4 Months, but the reproductive success of this crayfish is higher in wetlands with longer hydroperiod, that are more easily and stably invaded (Cruz and Rebelo, 2007; Gutiérrez Yurrita and Montes, 1999); unfortunately, deep and permanent wetlands are also the ones hosting the richest odonate communities (Tables 4, 5, and 6), while most of species can not successfully reproduce in ephemeral wetlands (Heidemann and Seidenbusch, 2002; Schultz, 2009; Stevens and Bailowitz, 2008). Our study therefore suggests that odonates can be extremely sensitive to the impact of this AIS. Larvae and exuviae are the stages that are affected first by the crayfish and show the earliest crayfish impact at the wetland scale, while negative effects on adults are less evident at early stages of the crayfish invasion and may indicate long term consequences and landscape scale effects.

The conservation of odonate communities requires management actions aimed to limiting the distribution of *P. clarkii;* in invaded regions keeping isolated wetlands free from the invasive crayfish, and create new breeding sites, can be effective conservation tools. Unfortunately, the control of this AIS is extremely difficult (Aquiloni et al., 2010; Gherardi, 2006; Gherardi and Panov, 2009), challenging conservation of freshwater biodiversity in the quickly expanding invaded areas.

6. Conclusions

Alien species invasions are an increasingly important aspect of global change (Didham et al., 2007; Perrings et al., 2010) but the managing of AIS and the detailed assessment of their impact on the invaded ecosystems is challenging; in particular, there is a lack of detailed information on AIS invasion processes and damages.

This research analyses different aspect of the *P. clarkii* invasion, starting from the spatial patterns and processes determining its invasion (chapter 2), and subsequently studying its impact on native freshwater populations (chapters 3, 4, and 5). I have chosen to investigate amphibians and odonates because they are well known taxa, already widely used in environmental research, and for their ecological, cultural and conservation value. I analysed a large area, sampling a total of 148 wetlands, including running and standing waters both temporary and permanent, where *P. clarkii* is at the early stages of the invasion, with invaded wetlands alternate to not invaded suitable wetlands (chapter 2). The data analysis considered environmental parameters of each wetland and of the surrounding landscape. The spatial autocorrelation was an additional parameter evaluated in all our analysis.

Procambarus clarkii is an AIS with high tolerance to a wide range of environmental conditions, therefore some authors suggested that habitat characteristics can only marginally determine its invasion trend (Cruz and Rebelo, 2007; Gherardi, 2006). Nevertheless, my data (chapter 2) on the early stages of invasion showed that, at this phase, habitat characteristics can be extremely important drivers of the invasion, in particular my results indicate that, at early invasion stages, *P. clarkii* spread is strongly associated with large and permanent wetlands in human-altered landscapes. In addition, the presence of occupied wetlands within 2500 m increases the likelihood of occurrence of *P. clarkii*, highlighting the importance of dispersal processes for invasion dynamics. These environmental requirements should be considered during the management of this AIS.

Furthermore, this research highlights the importance of studying the early phases of an invasion, confirming that, in this phase, the AIS populations have higher environmental requirements: in fact, the knowledge of environmental features that can

strongly determine AIS success as invaders might make them more vulnerable at management actions.

This research confirms the association between *P. clarkii* and human activities (chapter 2); *P. clarkii* is an omnivorous feeder with high tolerance to human disturbance, its populations were associated with human-altered landscapes; altered environments with simplified communities can favour the invasive crayfish that, after reaching high population densities in disturbed environments, can disperse to more natural biotopes (Correia 2003; Didham et al. 2007; Cutway and Ehrenfeld 2009).

AIS can strongly stress the invaded environments and make them unsuitable for native species (Didham et al., 2007; Strayer, 2010); but only a small subset of studies considered the effects of AIS concurrently to the ones of environmental modifications (Didham et al., 2007); in fact the impact of AIS on native communities is strongly negative (paragraph 1.2) but the dramatic effects on native ecosystems have multiples causes and mechanisms that, at present, are poorly understood. The mechanisms determining the observed negative effects, and not only the negative effects, must to be considered for AIS management: this is especially true during the control phase of AIS management (Cox, 2004; Polasky, 2010) and helps to set out more effective management actions.

Procambarus clarkii has a strong impact on both amphibian and odonate communities (chapters 4 and 5) (Cruz et al., 2008; McCarthy et al., 2006); this research confirmed its dramatic negative impact and I investigated the mechanisms determining it. Both amphibians and odonates have complex life cycles and studies on main life history stages give the opportunity to observe the AIS impact from different perspective (Córdoba-Aguilar, 2008; Wells, 2007). Separate analysis of different life stages led to different estimates of the invasive crayfish impact. This research (chapters 3, 4, and 5) shows that *P. clarkii* impact on native communities determines loss of reproductive sites and fall down of the reproductive success when native populations of amphibians and odonates use the invaded sites for breeding, and the impact of *P. clarkii* could be even more negative when invasion occurs in human dominated areas where suitable breeding

sites are already scarce, and where natural populations are affected by multiple human-related stressors that enhance the AIS negative effect (Didham et al., 2007; Perrings et al., 2010).

All the considered taxa (the invasive crayfish, amphibians, and odonates) are strongly influenced by environmental features. This research underlines that, while the invasive crayfish is associated to human-altered landscapes, odonates and amphibians find their optimal habitat in more natural environmental conditions; considering the wetland features that are optimal for the invasive crayfish and for native communities, this research shows further important differences mainly referred to parameters like the wetland size, depth, hydroperiod, exposition, and presence of aquatic macrophytes (chapters 2, 3, and 5).

This research suggests the importance to well understand the habitat requirements of both AIS and threatened native communities to perform effective management actions (chapters 2, 3, 4, and 5).

AIS are currently the main cause of biodiversity loss both at local and at global scale, but multiple processes have a joint action in determining the invasion dynamics and impact on native ecosystems (Didham et al., 2007; Strayer et al., 2006). Prevention of new introductions is certainly the best strategy to tackle the AIS problem (paragraphs 1.2 and 1.3), but when prevention fails, the early detection of an invasive species and the rapid application of control strategies are the decisive actions to limit the impact of exotic species (Hulme et al., 2009; Perrings et al., 2010; Wittenberg and Cock, 2001).

This research helps to identify the ongoing processes at early stages of the AIS invasion and the consequences that the invasive crayfish will have in the near future; these information may allow managers to start conservation actions before than consequences of AIS become irreversible.

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