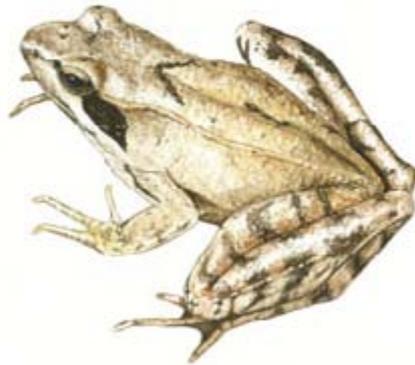


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**Corso di Dottorato di Ricerca in Scienze Naturalistiche e Ambientali, XVII ciclo**

**DISTRIBUTION AND DIVERSITY OF THE SEMI-AQUATIC  
HERPETOFAUNA IN THE RIVER PO BASIN:  
FROM THE MICROHABITAT ANALYSIS  
TO THE REGIONAL PERSPECTIVE**

**Tesi di Dottorato di:  
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**Anno Accademico 2004-2005**

Cover illustration: Micol Farina.

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# 1. INTRODUCTION: ECOLOGY AND CONSERVATION OF SEMI-AQUATIC HERPETOFAUNA

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In the last 15 years, there has been a worldwide concern about the widespread amphibian decline. Since the early 1990's the extinction or the decline of a large number of amphibian populations has been reported in all the continents, and also in habitats apparently without human disturbance, such as the the tropical rainforests (e.g., Barinaga, 1990; Wake, 1991, Blaustein and Wake, 1995; Lips, 1998; Houlahan *et al.*, 2000, Green, 2003; Lips *et al.*, 2003; 2004). Thus, a great number of studies tried to assess the causes of this generalised decline. It is now evident that a large number of factors are concurring in determining the amphibian decline; for example, the spread of a variety of pathogens, (including viruses, bacteria, parasites, protozoans, oomycetes and fungi) affected a number of populations. Among others, Lips and co-workers (2004) observed in some localities of Central America a decline of 90-99% of amphibian populations since the 1970's: this decline is in large part imputable to the fungus *Batrachochytrium dendrobatidis*, a species only recently discovered (Longcore *et al.*, 1999), that is likely responsible of amphibian decline also in several other countries (Berger *et al.*, 1998; Kiesecker *et al.*, 2004). Moreover, the same environmental alteration can have different consequences on different populations. For example, it is likely that climatic oscillations caused the extinction of the toad *Bufo periglens* from the tropical rain forest of Costa Rica (Pounds and Crump, 1994). Conversely, north American amphibian populations of Rocky Mountains did not appear to be affected by climatic change (Blaustein *et al.*, 2002). The increasing ultraviolet-B (UV-B) radiation caused by the depletion of stratospheric ozone is lowering the hatching success of a number of species, and it induces sublethal effects that may affect fitness related traits such as behaviour, development, physiology and anatomy (Blaustein *et al.*, 2001) . However, the susceptibility to UV-B radiation can be different among species, since some species have behavioural, physiological and molecular defences against the UV-B radiation (Blaustein and Belden, 2003).

Moreover, the interactions among environmental alteration, introduction of diseases, increase of UV radiation and invasion of exotic organisms threaten more strongly the populations than do the effect of a single factor (Blaustein and Kiesecker, 2002; Kiesecker *et al.*, 2004). Finally, also within a species, different populations can have local adaptations that increase or reduce the negative effects of environmental alteration (Belden and Blaustein, 2002). In general, the complexity of these phenomena make it difficult to evaluate how a population can be affected by environmental alterations and thus to suggest management actions.

The concern about the amphibian decline results in an increasing number of management plans, and in the protection of many species. In the European Union, within the mainframe of the Habitat Directive (EC 1992/43) 23 amphibian species "require the designation of special areas

for their conservation” (EC 1992/43, enclosure B). In proportion to the number of species living in Europe, this number is proportionally larger than the number – for example - of mammal species: only 33 species of terrestrial mammals are included in the same enclosure, despite in Europe much more mammal than amphibian species live (~ 120 species of mammals, and ~ 60 species of amphibians), and despite the interest about the large mammals is frequently higher, since mammals are more attractive to the people and thus are used as “flagship species”. Thus, the delineation of factors leading to the amphibian decline have a pivotal role in the planning of their conservation and management.

Habitat alteration is an extremely important cause of amphibian decline in the most anthropic areas. In temperate areas, most of amphibians has a complex life-cycle, with aquatic larvae and terrestrial or semi-aquatic adults. Thus, these species require both wetlands for their reproduction, and natural upland habitats for the post-reproductive stage. Moreover, for these animals linkages in the landscape are required to allow movements between the reproductive and the post-reproductive habitats (Marsh and Trenhman, 2001) In practice, most amphibian species are small and have very scarce mobility: the longest recorded terrestrial migration is ~ 10 Km (Marsh and Trenhman, 2001; Semlitsch and Bodie, 2003). Thus, the survival of amphibian populations require a suitable terrestrial environment adjacent to waterbodies suitable for the reproduction (Guerry and Hunter, 2002). Moreover, amphibian populations frequently survive as a metapopulation (Sjorgen, 1991; Hanski and Gilpin, 1997; Marsh and Trenhman, 2001). Within a metapopulation, there is a flow of individuals among subpopulations: each subpopulation can become extinct for catastrophic events, for habitat unsuitability or for stochastic factors, but the flow of individual allow the (re)colonization of unoccupied patches if they become suitable. Thus, within a metapopulation there is a dynamic equilibrium of extinctions and colonisations, allowing the long term survival of species (Sjorgen, 1991). Conversely, without flow of individuals among populations, each subpopulations can become extinct for habitat alteration, stochastic or catastrophic events or for inbreeding depression (Hanski and Gilpin, 1997; Hanski, 1998; Saccheri *et al.*, 1998).

Thus, the study of the ecology of amphibian populations for their conservation can not be performed without considering the complex relationships among the different features of environment at different spatial scales. For example, chemical and physical features, plant and animal communities of wetlands are very important for amphibian reproduction, but a population can not persist without a suitable upland habitat; similarly, a suitable upland habitat does not allow the persistence of a population without a suitable wetland. Moreover, upland and wetland habitats can not be considered to be independent, since there is a strong influence between them, for example for the flow of biomass and of nutrients (Kiffney *et al.*, 2003; Pace *et al.*, 2004).

At a larger spatial scale (landscape scale), a patch suitable for an amphibian population, holding both a suitable aquatic and terrestrial habitat, can be isolated from other patches. Thus, if the patch is occupied by a population, this population face off the risk of loss of genetic diversity and thus of inbreeding depression (or genetic load); in turn, the loss of genetic diversity and the subsequent loss of fitness of individuals can cause an high risk of extinction of population (Saccheri *et al.*, 1998; Rowe *et al.*, 1999; Rowe and Beebee, 2003; see also Hedrick, 2001; Keller and Waller, 2002).

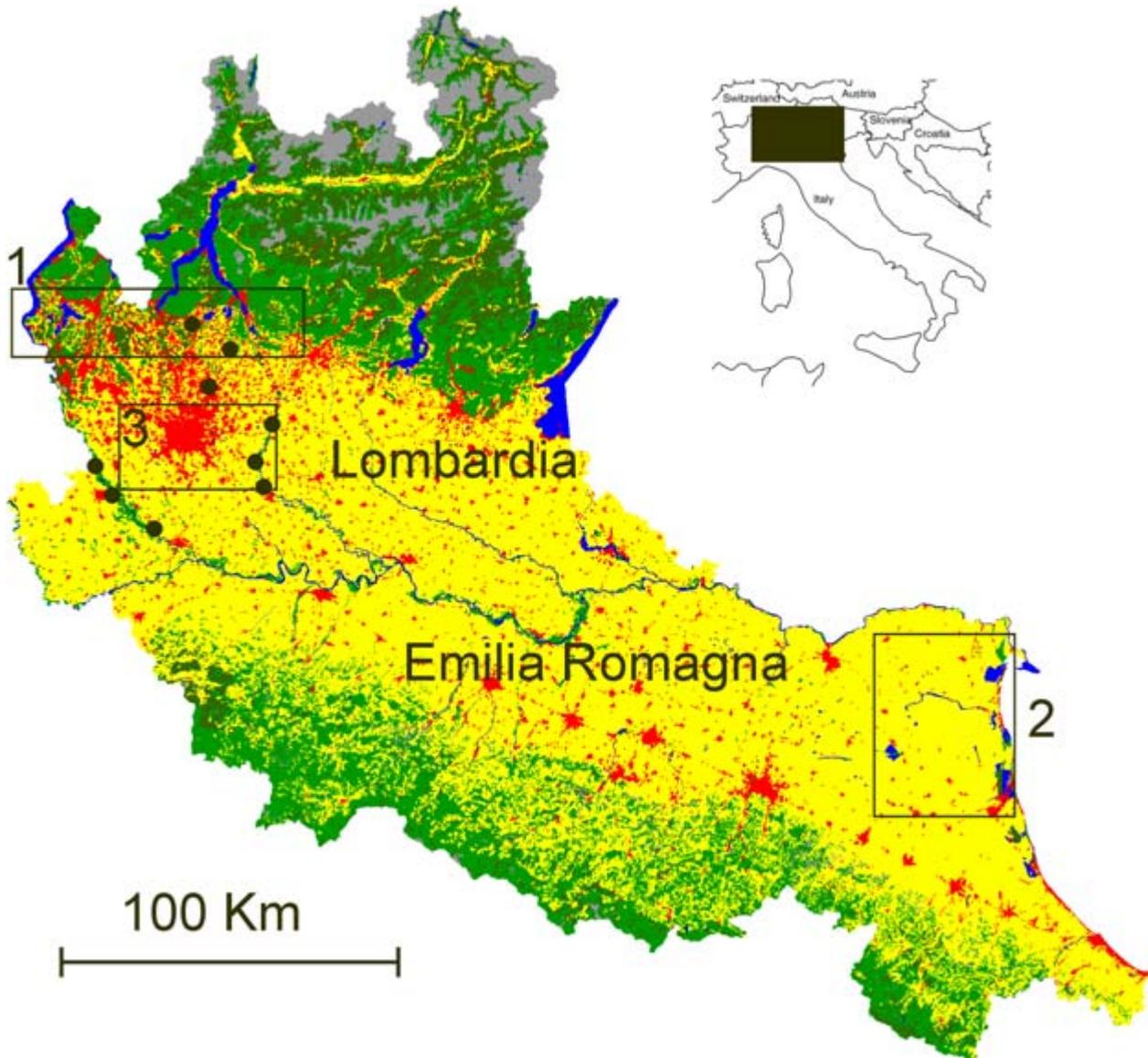
At regional scale, amphibian populations can quickly evolve and adapt to local conditions, such as differences among localities in climate, altitude or biotic communities (Merila *et al.*, 2000; Loman, 2002 a; 2002b; Laugen *et al.*, 2003a; Skelly, 2004). For example, Skelly and Freidenburg (2000) observed that the natural come back of beaver *Castor canadensis* caused differences among wetlands in sun exposure: the beaver cut the small trees surrounding the wetlands, thus the wetlands occupied by the beaver became more sunny and warmer. In turn, these thermal differences among wetlands caused genetic differences in thermal optima and development rate in the tadpoles of the wood frog *Rana sylvatica*: these differences among populations likely evolved quickly, in less than 30 years (Skelly and Freidenburg, 2000; Freidenburg and Skelly, 2004; Skelly, 2004). Thus, differences in the interactions between amphibians and environment can be present also for populations that are only a few Kilometres apart (Skelly 2004).

In summary, these considerations suggest that: (1) the study of relationships between aquatic and terrestrial habitat allow to evaluate the habitat required for the persistence of a population; (2) the analysis of effect of spatial configuration of patches (i.e., relative isolation) on inbreeding depression and extinction risk allow to evaluate the consequences of interruption of a metapopulation system on the subpopulations; (3) the analysis of intraspecific variation at regional scale allow to evaluate if differences among populations in the evolutionary history or in the interaction with other biotic or abiotic factors can have consequences for the regional conservation plans in a long term perspective.

Similar considerations can be done for other components of semi-aquatic herpetofauna, such as turtles, which need upland habitat for reproduction and wetlands for the adults (Beebee, 1992; Mazzotti, 1993; Gibbons, 2003; Semlitsch and Bodie, 2003).

An exhaustive study about the ecology of semi-aquatic herpetofauna should kept in count all these complex interactions that can happen at different spatial scales. However, these phenomena are complex and not easy to investigate: in practice, much of studies is performed at only one of these spatial levels.

The aim of this thesis was to study semi-aquatic herpetofauna in the River Po Lowland and on the adjacent hills, and to evaluate the complex relationships between species distribution, environmental alteration, population fitness and local adaptation. Most of this research has been performed in the western Lombardy (provinces of Milano, Lecco, Como, Pavia, Varese and Lodi, Fig. 1). For the European pond turtle *Emys orbicularis*, I analysed also wetlands belonging to the River Po Delta (eastern Emilia-Romagna: fig. 1; Cap. 3). Western Lombardy is one of European areas with the highest human presence and development in Europe (Provincia di Milano, 2003), but it still hold some natural areas, mainly in the hills and along the main rivers, frequently within regional parks. In these areas still survive the amphibian species related to lowland environments: in Italy, lowland species are among the most threatened amphibians (Andreone and Luiselli, 2000), since in lowlands the exploitation of landscape and wetlands has been very strong since many centuries. For example, the Italian agile frog *Rana latastei* live in this area: this frog is a threatened species endemic of padano-venetian lowlands (see Cap. 5). Indeed, the contemporary presence of natural patches within a human dominated landscape make this area ideal for the aims of this study, since the human modified areas can be compared with the most natural ones.



**Figure 1.** Study area. 1: hills of western Lombardy (see also chapter 2); 2: River Po Delta (see also chapter 3); 3: lowland area surrounding Milan (see also chapter 4); black dots: populations of *Rana latastei* sampled (see chapters 6-9). The map is derived from the CORINE land cover: in red, urban areas; yellow: cultivated areas; green: natural vegetation (mainly woodlands); grey: sterile areas; blue: water bodies.

### Structure of the thesis

This thesis is divided in two main sections, to better analyse the complex interactions at the three spatial scales considered.

The first section (chapters 2-4) dealt mainly with the relationships between distribution of semi-aquatic herpetofauna and habitat features. I evaluated in three different systems (hill streams; wetlands of Po River Delta; wetlands surrounding the city of Milan) if the presence/absence of species, their abundance and the structure of communities is related to environmental features, measured at the microhabitat scale (wetland features) and at the landscape scale. If the number of species present was large enough, I performed the analysis at

the community level (chapter 4). Otherwise, (chapters 2-3) the analysis focused on only one species. In these cases, I analysed more thoroughly the relationships between the autoecology of each species and habitat. In this section, I also analysed the distribution of a reptile, the European pond turtle *Emys orbicularis* (chapter 3). Despite this turtle obviously is not an amphibian, it has semi-aquatic habits. Semi-aquatic reptiles share with amphibians an high conservation priority, since environmental alterations are causing their decline in a similar fashion (Beebee, 1992; Mazzotti, 1993; Gibbons, 2003; Semlitsch and Bodie, 2003). Thus, it can be interesting to evaluate how species belonging to different taxa share similar threats, since they have similar habits.

The second section (chapters 5-9) focus on only one species, *R. latastei*. This species has limited mobility, small geographic range and lives only in non exploited lowland habitats (lowland forests: see chapter 5). By focusing on only one species, I evaluated at regional scale how differences among sites where the populations live influence the breeding ecology, the fitness and the life-history traits of a threatened species. I measured (1) if the presence of a closely related frog species, the agile frog *Rana dalmatina*, causes interference with the breeding biology of *R. latastei* (chapter 6). Observing this relationship would indicate that the presence of reproductive interference can be an important factor for the pattern of distribution of this species. (2) I evaluated if the isolation of some population, caused by landscape alteration (interruption of connectivity among patches; presence of barriers) causes differences in fitness related traits among populations. For this aim, three populations that are now completely isolated from conspecific populations by human barriers are compared with six populations belonging to large metapopulation systems (chapter 7). (3) I evaluated if populations living in different areas of the Lombardy (lowland vs. foothill) have evolutionary different histories, and thus represent different evolutionary units (chapter 8). Intraspecific diversity is recognised as a central conservation problem (Huges *et al.*, 1997; Sinclair, 2001), and the potential for a species evolutionary success is maximized through the maintenance of adaptive diversity (Frankel, 1974; Hedrick, 2001). Thus, intraspecific diversity should be kept into account in evaluating the relationships between species and habitats (Skelly, 2004) and in regional relocation programs. Finally, revealing the natural mechanisms that allow the maintenance of genetic diversity among populations can be an important task for the species persistence in a long term perspective (chapter 9).

The chapters 2-9, with the only exception of chapter 5, are partially independent papers. Some of these has been published or are in press, while most are going to be submitted. All these chapters can be read independently, as they have an introductory paragraph reporting the theoretical mainframe of the topics discussed within the paper. The only differences between these chapters and the published version is their format and the reference section, since all the references has been merged at the end of the thesis. However, these chapters can also be viewed as an attempt to move from the microhabitat to the regional perspective, and to show, within the same landscape, the relative importance of forces acting at different spatial scale, from breeding habitat to evolutionary constraints. This integration across spatial and temporal scales is needed if we want a theoretical and empirical framework for the conservation of semi-aquatic herpetofauna in human dominated landscapes.



## 2. DOES THE INTERACTION BETWEEN UPLAND HABITAT AND WATER FEATURES INFLUENCE THE DISTRIBUTION OF FIRE SALAMANDER *SALAMANDRA SALAMANDRA*?

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### Abstract

Following the concern about the global amphibian decline, many studies evaluated the relationships between environmental alterations and species distribution. Much of them focused only on the aquatic or only on the upland environment; only a few dealt with amphibians breeding in streams, such as the fire salamander. We evaluated the relationships between salamander reproduction in 38 streams, the environmental features of upland environment measured in a buffer zone of 500 m, and some chemical and physical features of water. Two alternative models explain the salamander distribution. According to the first one, *S. salamandra* breeds in permanent streams with low phosphates concentration; according to the second one *S. salamandra* breeds in permanent streams surrounded by woodlands. The second model explain more deviance, moreover, phosphate concentration is positively related to the proportion of anthropogenic landscape, and thus negatively related to woodlands. Woodlands seem to influence salamanders directly since it is the habitat of adults. Moreover, the scarcity of human activities in wooded areas allow more suitable water features for larvae. We outline the importance of natural upland habitats for the conservation of semi-aquatic organisms.

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### Introduction

Since the claims of global amphibian decline in the early 1990's (Barinaga, 1990; Wake, 1991), a large number of studies investigated the causes of amphibian disappear. The factors that has been hypothesised as causes of amphibian population decline are: habitat destruction and alteration; global environmental change (e.g. climate change or increased UV-B radiation); diseases; contaminants; introduced species (see Beebee, 1996; Scoccianti, 2001; Blaustein and Kiesecker 2002 and references therein). Unfortunately, with an increasing number of studies on the amphibian decline, there has been little consensus on the causes. Despite the recognition that interaction among factor and context dependency can play an important role in the understanding decline, research programs typically focused on the direct effects of single factors (Alford and Richards, 1999; Blaustein and Kiesecker, 2002).

Many amphibian species have aquatic larvae and terrestrial adults. Thus, they experience both aquatic and terrestrial stressors: for the survival of a population, an aquatic habitat suitable for the reproduction should be adjacent to a terrestrial habitat. However, most of studies on the relationships between habitat alteration and amphibian distribution focused only on the breeding wetlands, with the unexpressed hypothesis that amphibian population dynamics result mainly

from processes occurring at breeding ponds (Marsh and Trehman, 2002). Since the adults of many amphibian species spent at the breeding wetlands only a few days or hours, living otherwise in terrestrial environment, the composition and configuration of upland environment play a pivotal role for the survival of populations (Guerry and Hunter, 2002). Indeed, the importance of upland environment for amphibians ecology could be not limited to their role as terrestrial habitat. Recent studies outlined that adjacent upland and wetland habitats are strongly interdependent, since there is an important flow of nutrients and biomass across these components of the ecosystems (Kiffney *et al.*, 2003; Pace *et al.*, 2004 and references therein). The interaction between upland and wetland component of ecosystems can be also stronger for the small waterbodies that are frequently used for the reproduction by amphibians.

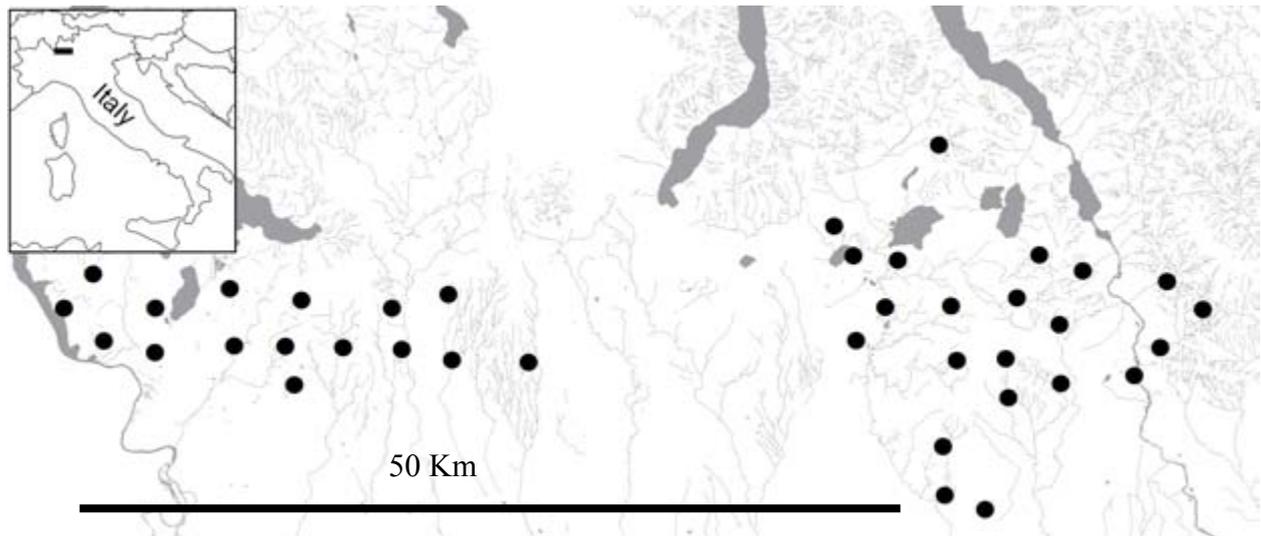
Streams are a second frequently overlooked habitat that has a pivotal role for some amphibian species. Most amphibians breed in pools, ponds or small lakes. However, running water is the breeding habitat of some amphibian species: in Europe, the newt of genus *Euproctus*, the salamanders *Salamandra salamandra* and *Salamandrina terdigitata*, the frogs *Rana pyrenaica*, *R. italica* and *R. graeca* breed preferably in running water. The scarce attention toward streams as amphibian habitat is showed by the excellent review of Marsh and Trenhman (2002): through their papers, the authors repeatedly ask “do amphibian population dynamics result from processes that occur at breeding pond?” and again “is pond isolation important to pond use or population persistence?”. These phrases suggest that the majority of studies about amphibian breeding sites investigated only still water wetlands.

The aim of this study was to evaluate the relative importance of aquatic and terrestrial habitat for the distribution of the fire salamander *S. salamandra* (Linnaeus, 1758). This species breeds mainly in running water, such as first order streams, while the adults spent their lifetime in wooded habitats (Lanza, 1983). Thus, it is likely that both terrestrial and aquatic habitat are important for *S. salamandra*. We also investigated whether the relationship among water features and features of terrestrial habitats are important to explain the distribution of this species.

## Materials and methods

### *Study area*

We investigated an area in the hills of western Lombardy (Fig. 1). By using the map of Lombardy, 38 first order streams were randomly selected in an area spanning for ~ 70 km from east to the west, in the provinces of Varese, Como, Lecco, Milano and Bergamo. Selected first order stream had an altitude comprised between 200 and 500 m: in Lombardy, *S. salamandra* live currently at an altitude > 200 m (Di Cerbo and Razzetti, 2004); the maximum altitude was limited to 500 m to avoid the presence of a strong gradient of altitude. The minimum allowed distance between two selected streams was 3000 m. We chose this distance to minimise spatial autocorrelation among sites, and because salamanders only rarely move more than 500 m from the breeding wetland (Semlitsch and Bodie, 2003), thus it is likely that different streams host different salamander populations.



**Fig. 1.** Study area. Black dots represent the 38 study sites; grey lines represent the hydrographical network.

## Methods

From April to early June 2004, each stream was visited at least three times, to evaluate the presence of salamanders. In each survey, the presence of salamanders was assessed by visual transects along the water courses, by repeated deep-netting of the stream bottom, and by use of funnel traps (Heyer *et al.*, 1994). The open side of funnel traps (diameter:10 cm) was placed toward the stream flow, mainly in pools; for each stream, three funnel traps were placed in different pools, the traps were checked the seventh day. The sampling effort seems to be adequate to evaluate the presence of salamanders without the risk to not detecting present salamanders: for each of six streams, two additional surveys were performed. If fire salamanders were present in a previous survey, we always re-detected them in at least one of these two additional surveys; we never detected salamanders in streams in which they were not detected in previous surveys. We collected a 0.5 litre water sample from each wetland in late May-early June for chemical and physical analysis. In laboratory, we measured water conductivity, concentration of nitrates and of phosphates. The concentration of oxygen was measured in the field using a digital probe. Two streams completely dried prior to our water sample collection, thus we analysed water samples from only 36 streams. The percentage of cover of upland habitat in a radius of 500 m from the stream was measured by using the GIS ArcView 3.2, on the basis of DUSAF land cover of Lombardy.

### *Data analysis*

Prior to perform the analysis, we tested for multicollinearity using the correlation matrix among independent variables. Since in all pairwise correlations  $|r| \leq 0.7$ , we assumed the regression models to be not biased (Berry and Feldman, 1985). We used logistic regression to relate presence/absence of salamanders to habitat features; we used the likelihood ratio to evaluate the significance of each variable, a variable being considered significant if it significantly reduces

the log-likelihood of the model (Menard, 1995). We build all the possible models in which all the predictors were significant, thus the models were ranked according their Akaike's Information Criteria (AIC). The model with the lowest AIC value is assumed to be the one explaining most of the variation using the fewest parameters; however, when candidate models deviated by only a few units of AIC, the biological relevance of the models should also be considered (Burnham and Anderson, 1998). Thus, we discussed the relative importance of models also in light of their ecological interpretation. We used Hosmer and Lemenshow  $r^2$  ( $R^2_L$ ) as a measure of percentage of deviance explained by the models.  $R^2_L$  can be considered a good analogue of  $r^2$  of linear regression for logistic regression (Menard, 1995). Percentage data were arcsine-square root transformed prior to analyze (Sokal and Rohlf, 1995); after transformation, we did not observe significant deviation from the parametric assumptions of the models.

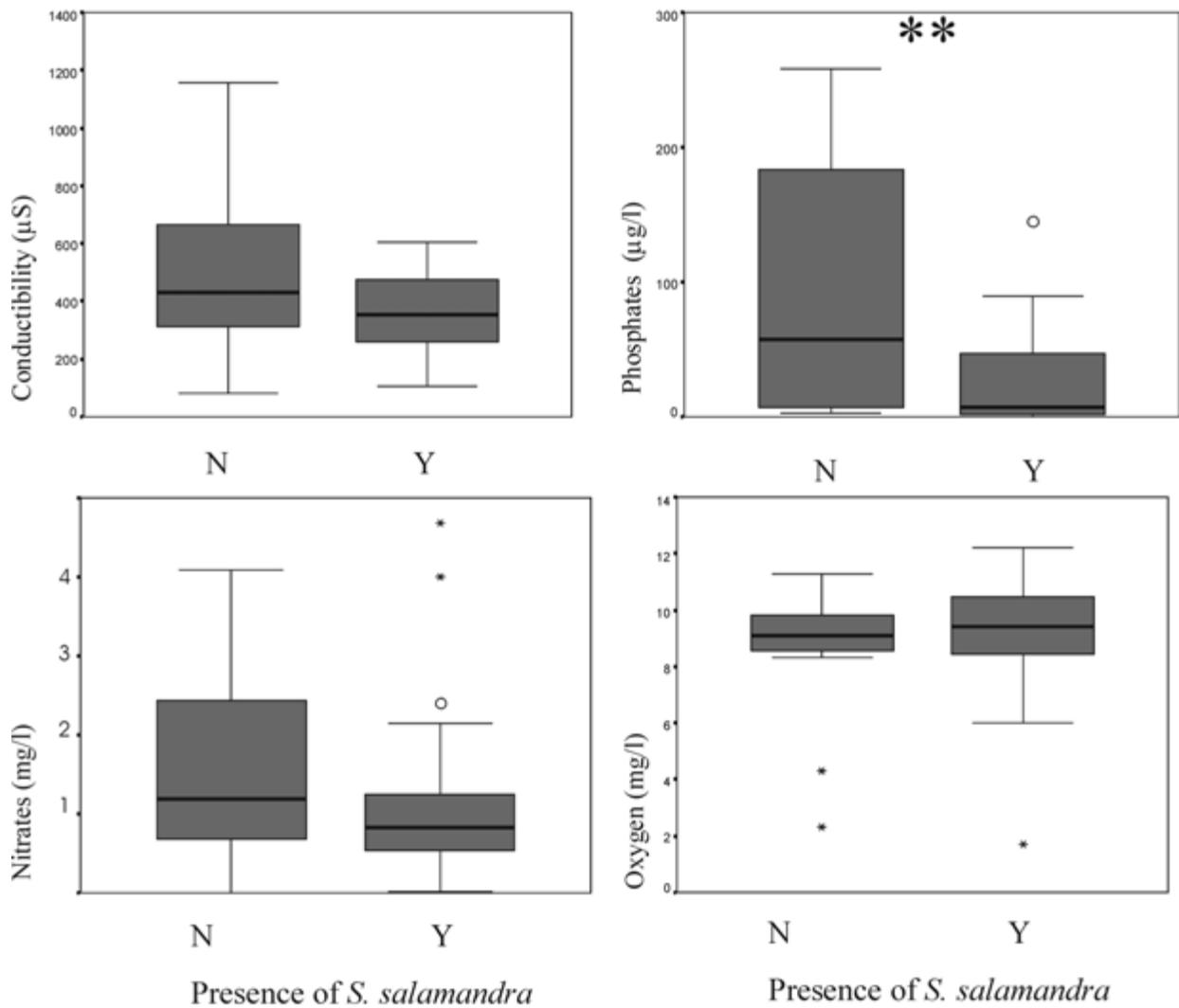
Model rank	Terms in model	$\beta$	$\chi^2$	Remove $P$	Model $\chi^2$	Model $P$	Model $R^2_L$	Model AIC
1	<b>Water permanence</b>	10.391	7.203	<b>0.007</b>				
	<b>Wood %</b>	10.075	14.26	<b>0.0002</b>				
	Constant	-16.665			18.781 (df=2)	<b>&lt;0.0001</b>	0.417	30.223
2	<b>Water permanence</b>	9.909	6.728	<b>0.009</b>				
	<b>Phosphates</b>	-0.016	8.092	<b>0.004</b>				
	Constant	-8.123			12.610 (df=2)	<b>0.002</b>	0.280	36.394
3	<b>Phosphates</b>	-0.014	6.991	<b>0.008</b>				
	Constant	1.339			6.991	<b>0.008</b>	0.155	44.101
4	<b>Water permanence</b>	8.940	8.174	<b>0.004</b>				
	Constant	-8.202			8.174	<b>0.004</b>	0.160	46.808
5	<b>Wood %</b>	5.712	8.051	<b>0.005</b>				
	Constant	-3.796			8.051	<b>0.005</b>	0.158	46.931

**Tab. 1.** Logistic regression models relating *S. salamandra* presence/absence to habitat features. Degrees of freedom = 1 if not otherwise specified.

## Results

Out of 38 streams surveyed, we observed larvae of *S. salamandra* in 23 sites (60.5%). We did not observe significant differences among occupied and unoccupied streams for conductivity ( $t_{14.157} = 1.349$ ,  $P = 0.198$ ), nitrates ( $t_{34} = 0.915$ ,  $P = 0.367$ ) or oxygen concentration ( $t_{34} = -0.757$ ,  $P = 0.454$ ). However, the streams occupied by *S. salamandra* had significantly lower phosphates concentration than the unoccupied ones ( $t_{34} = 2.835$ ,  $P = 0.008$ ) (Fig. 2).

We build five possible significant models (Tab. 1). According to both AIC and  $R^2_L$ , the best model (model 1) included as predictor % wood cover and water permanence: this model suggest that salamanders use for reproduction permanent streams surrounded by woods. Out of the four models with higher AIC, two (model 4 and model 5, tab. 1) are subsets of model 1.

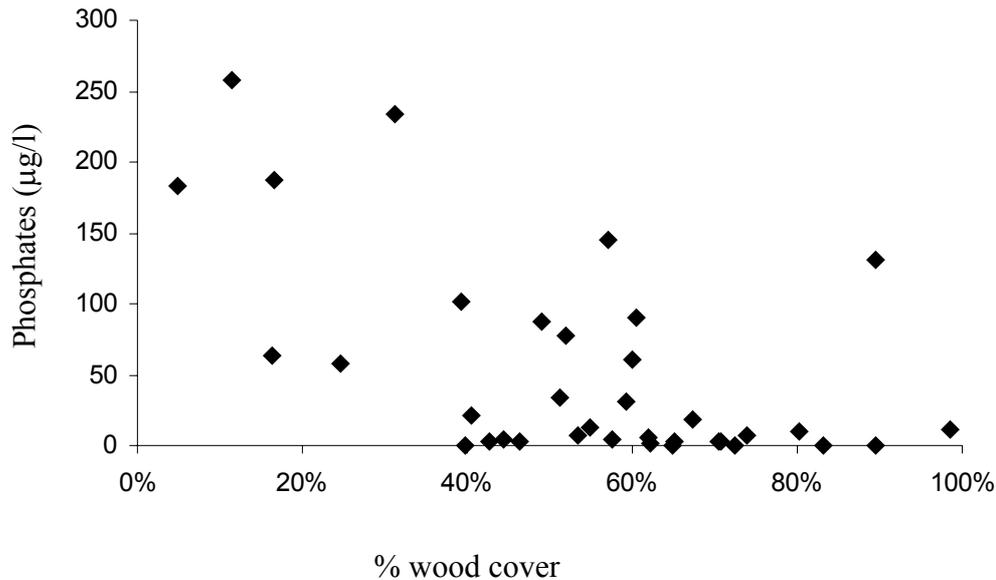


**Fig. 2.** Boxplots of water conductivity, phosphates nitrates and dissolved oxygen concentration in streams occupied and unoccupied by *S. salamandra*. \*\*: significant differences in phosphate concentration between occupied and unoccupied streams.

However, the model with the second best AIC value (model 2) included as predictors phosphate concentration and water permanence, suggesting that salamanders use for reproduction permanent streams with low phosphate concentration. Indeed, the AIC of model 2 is higher than those of model 1, suggesting that model 1 perform better. It should also be noted that phosphate concentration and % of wood cover are strongly negatively related (Pearson's correlation:  $r = -0.598$ ,  $P < 0.0001$ ): the streams surrounded by woods have lower phosphate concentration (Fig. 3).

## Discussion

The aim of this study was to evaluate the relative importance of terrestrial and aquatic habitat for the distribution of reproductive sites for salamanders. Indeed, our results are apparently inconclusive: out the two best model, the first one (model 1) suggested that-terrestrial



**Fig. 3.** Relationship between % wood cover in the 500 m surrounding a stream and phosphate concentration of water.

environment was the most important predictor of salamander distribution, while the second, the third and the fourth ones (models 2, 3 and 4) suggested that stream features are the most important. Indeed, the use of AIC allow to hypothesize that terrestrial environment could be most important than the aquatic one. Here we discuss if it is possible to use the ecological interpretation of our models to evaluate their relative importance.

#### *Models interpretation*

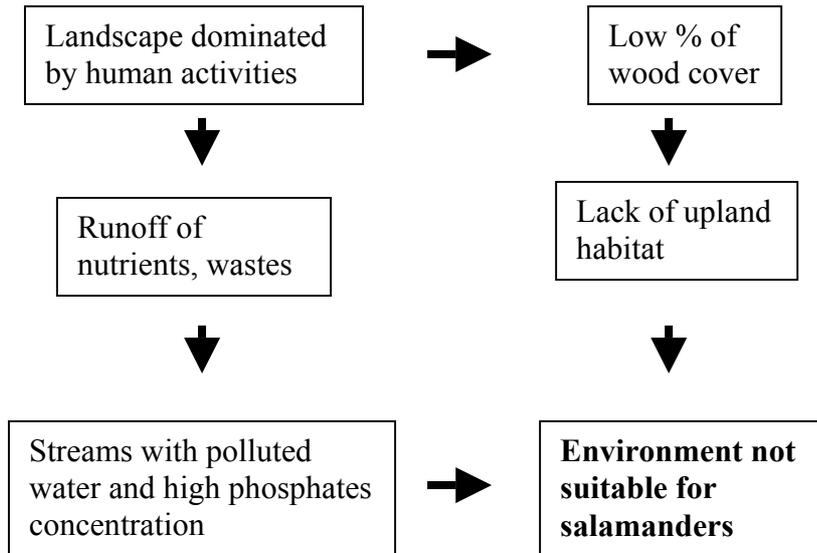
Three out of the five significant models (model 1, model 2 and model 4 in Tab. 1) outlined that water permanence during spring is important for the presence of salamanders. This result is not surprising, since in spring larvae of *S. salamandra* require up to four months for metamorphosis (Lanza, 1983; Nollert and Nollert, 1992). Some of our streams did not retain water during the overall period April – June, likely because their hydroperiod is strongly dependent from rainfall. Thus, these streams are not suitable for salamanders, since larvae within temporary watercourses have a great risk of dying prior to metamorphose. The presence of permanent water is important to allow the persistence of many amphibian species requiring long time for metamorphosis (e.g., Skelly *et al.*, 1999; Baber *et al.*, 2004). A number of studies observed that many amphibian species are associated to semi-permanent wetlands, since semi-permanent water bodies are unoccupied by predatory fish (Atkins, 1998; Kolozsvary and Swihart, 1999; Weyrauch and Grubb, 2004, see also chapter 4), Fish, such as trouts, are important predators for *S. salamandra* arvae (Lanza, 1983). However, first order streams that are the habitat of larval salamanders are infrequently inhabited by fish: the maximum depth of pools normally is only 5–10 cm, and most of streams have a depth of less than 1 cm. Indeed, During ~ 800 trapping days, we caught only one small fish in our funnel traps: this observation suggest that fish are very scarce in our first

order streams. Thus, *S. salamandra* is associated to permanent streams because in the permanent streams where it live fish are not present or are scarce.

The importance of woodlands for the distribution of reproductive sites of *S. salamandra* can be explained by their importance for the adults. Adult fire salamanders are strictly terrestrial, and only females move to the water to give birth to larvae (Lanza, 1983; Nollert and Nollert, 1992). Woodlands have a pivotal role for the persistence of many amphibian species, since they provide abundance of prey items, moisture and protection from dehydration, and a dense understory and leaf litter that can be used as shelter by adults (Lanza, 1983; Lemkert and Brassil, 2000; Curtis and Taylor, 2003; Shabetsberger *et al.*, 2004; Porej *et al.*, 2004; Weyrauch and Grubb, 2004). Moreover, woodlands can be important for the dispersion of adults, allowing a lower risk of death during migration and possibly the existence of metapopulations (Hanski and Gilpin, 1997; Di Cerbo and Razzetti, 2004). Their importance for the dispersion of *S. salamandra* can be also higher than for other amphibians because salamanders move over short distances (Bovero *et al.*, 2001; Marsh and Trenhman, 2001; Semlitsh and Bodie, 2003 and references therein).

The interpretation of phosphate concentration as a possible predictor of salamander distribution is more puzzling. It is possible that phosphates have per se a negative effect on water quality for salamanders. However, less direct interpretations regarding the relationship between phosphate concentration and salamander distribution can be hypothesized. First, high phosphates can be related to high organic matter content and to low oxygen levels, especially close to the bottom and during night time. Moreover, some macroinvertebrates do not live in streams with high nutrient contents (Rosenberg and Resh, 1992), thus, it is possible that such streams do not host some taxa important for the feeding of salamanders (i.e., Plecoptera, Gammaridae, Weitere *et al.*, 2004). Again, a high phosphate concentration could be the effect of urban waste in streams, or of the runoff of agricultural fertilisers from crops: thus, it could be an indirect measure of the presence of other pollutants not analysed in this study, such as pesticides or other chemicals. Finally, it should be noted that phosphate concentration is not independent from the land cover in the area surrounding the stream. Streams surrounded by a less natural landscape (i.e., low wood cover %) are those with higher phosphate concentration (Fig. 3). Indeed, if we consider the % of landscape occupied by anthropogenic activities as (% of landscape occupied by urban areas ) + (% of landscape occupied by crops) we observe that: (1) the % of anthropogenic landscape is negatively related to the % of wood cover ( $r = -0.904$ ,  $P < 0.0001$ ) and (2) the % of anthropogenic landscape is positively related to phosphate concentration ( $r = 0.518$ ,  $P = 0.001$ ). These observations confirm that phosphates concentration depend from the presence of human activities in the landscape, such as agriculture, and of human settlements.

In summary, a complex pathway can be hypothesized about the effects of human modification of landscape on the distribution of *S. salamandra* (Fig. 4). First of all, landscape alteration and subsequent loss of wooded upland habitat causes the loss of habitat for adults: the absence of salamanders from streams surrounded by anthropogenic landscape can be caused by the absence of a suitable upland habitat for adults. Moreover, anthropogenic landscape alterations influence the water features of small streams. Water features of small streams within



**Fig. 4.** Diagram of possible relationships among landscape alteration, upland and wetland habitat suitability, and distribution of *S. salamandra*.

human dominated areas are different from those of streams in more natural areas. In turn, these differences could cause lower suitability for aquatic larvae of salamanders, and streams within anthropogenic landscape can be not suitable as reproductive habitat of salamanders.

### *Conclusion*

Many studies relating the distribution of semi-aquatic fauna to wetland features have completely overlooked the complex influence that upland habitat have on these animals and on aquatic environments (Gibbons, 2003). However, recent studies outlined that the terrestrial environment surrounding wetlands have a pivotal role in the nutrient cycle and in the food web of wetlands. Using a large scale experiment, Kiffney and co-workers (2003) demonstrated that streams with a reduced wooded buffer zone have higher nitrate and phosphate concentration than do streams surrounded by a wider wooded area, and suggested that the wooded buffer zone have strong influence on light intensity, microclimate and food web of ecosystems. Using a whole-lake experiment, Pace and co-workers (2004) observed that ~ 50% of the carbon in the lake wood web come from the upland environment surrounding the lake. We hypothesize that the effects of upland environment on wetlands could be also stronger for the small environments that we considered, first order streams being normally less than 1 m across. Our results suggest that the alteration of landscape surrounding small streams can modify both the habitat of adult salamanders and the habitat of larvae. Therefore, a landscape alteration could quickly make an habitat unsuitable for salamanders, affecting both adults and reproductive output. The preservation of a wide terrestrial habitat surrounding small wetlands should be prioritised for the conservation of semi-aquatic organisms.

### 3. THE IMPORTANCE OF AQUATIC AND TERRESTRIAL HABITAT FOR THE EUROPEAN POND TURTLE (*EMYS ORBICULARIS*): IMPLICATIONS FOR CONSERVATION PLANNING AND MANAGEMENT

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*Canadian Journal of Zoology*, in press.

#### **Abstract**

The European pond turtle *Emys orbicularis* (Linnaeus, 1758) is threatened throughout its distribution, prompting management of habitats and populations for conservation. Quantitative data on habitat requirements for this species are needed to better evaluate what areas are the most suitable, or what actions can improve habitat suitability. We studied relationships between the distribution and abundance of *E. orbicularis* and the environment by analysing water quality and features of 39 wetland and upland habitat in the Po River delta of northern Italy; visual transects and point counts were used to determine turtle presence and relative abundance. *Emys orbicularis* occurs more frequently and abundantly in permanent wetlands surrounded by woodlands, and its presence does not appear to be related to water eutrophication. Woodlands strongly influence adjacent wetland features and may be important for turtle terrestrial activities such as nesting and dispersal. Habitat management and conservation plans for *E. orbicularis* should include protection of extensive terrestrial woodland habitat, containing diverse wetland systems, to support turtle survival at different life history stages. The requirements for wetlands and natural terrestrial habitat are difficult to meet in the current human-dominated European lowlands, nevertheless conservation plans for this species should take a broad-scale approach.

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#### **Introduction.**

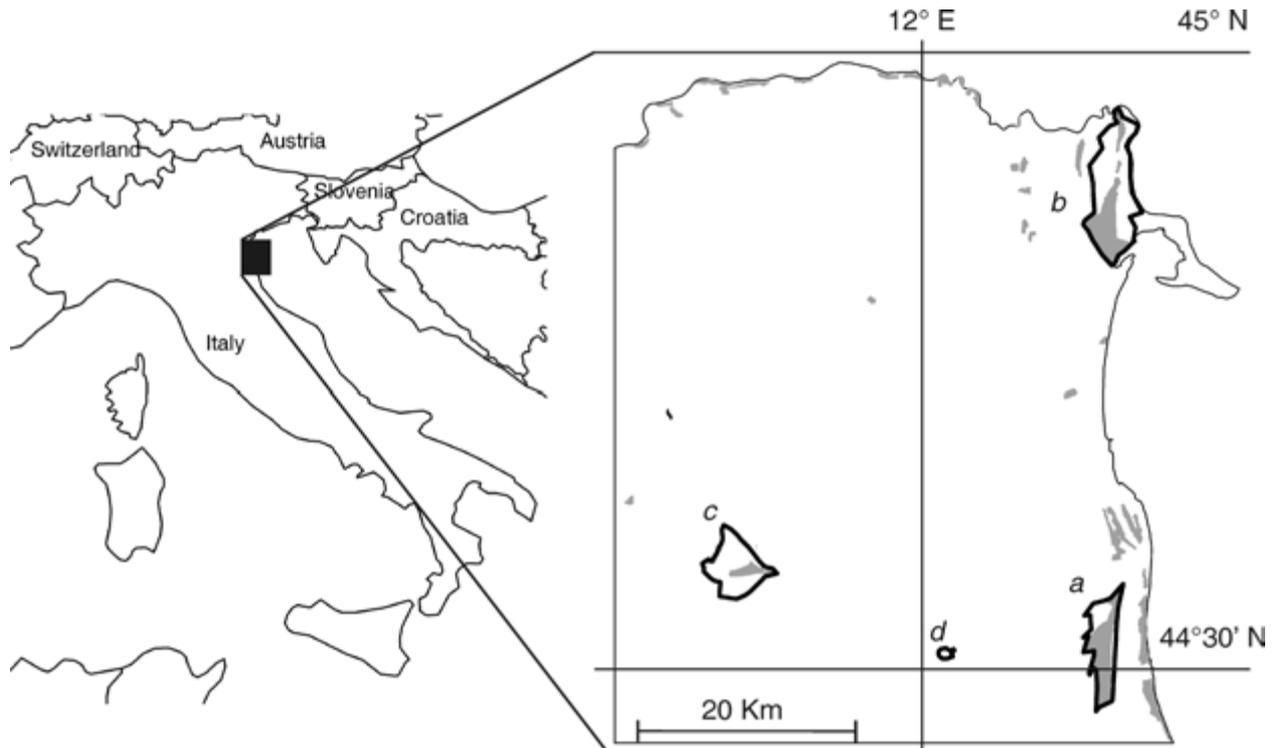
Many turtle species are of conservation concern worldwide, as they are threatened by human exploitation for food, pet markets, the shell trade, habitat deterioration and loss and competition with exotic species (Klemens, 2000). These problems are worsened because turtles have limited capability for rapid demographic recovery after decline, due to the relatively long time required for sexual maturity, to low reproductive output, high nest predation and juvenile mortality (Iverson, 1991; Congdon *et al.*, 1993). Therefore, many turtle species are protected and conservation plans are being implemented for some species. However, because financial resources for conservation are intrinsically limited, there is need for improved understanding of turtle ecology in order to optimise conservation results with currently available resources (Dalton, 2003).

The European pond turtle *Emys orbicularis* (Linnaeus, 1758) has the largest geographic range of any freshwater turtle in Europe, but has become rare in most of the countries where it occurs.

*Emys orbicularis* is rigorously protected in the European Union and the designation of special areas is required for its conservation (EU 1992/43). Thus, there is growing interest in the conservation of this species, as evidenced by several international symposia in recent years (see Fritz *et al.*, 1998; Buskirk and Servan, 2000; Fritz *et al.*, 2002). The decline of *E. orbicularis* is well documented but there is ongoing debate over the causes of these declines. The most widely suggested causes include: hunting for food; habitat loss from wetland drainage for agriculture; declining quality of residual habitats; water pollution; and competition with the introduced North American slider turtle *Trachemys scripta elegans* (Schneeweiss *et al.*, 1998; Ballasina and Lopes-Nunes, 2000a; Quesada, 2000; Cadi and Joly, 2003). Conservation actions to counteract the decline of this species, including recovery individuals confiscated from poachers, relocation plans, and habitat preservation and management, are ongoing in several European countries (Gariboldi and Zuffi, 1994; Delevaud *et al.*, 1998; Ballasina and Lopez-Nunes, 2000a; Lacomba and Sancho, 2000; Schneider 2000). However, to our knowledge, studies of *E. orbicularis* ecology have dealt with only one or few biotopes at a time, and frequently lack statistical analysis (Fritz *et al.*, 1998; Buskirk and Servan, 2000 and references therein). It is difficult to generalise the results of such studies to obtain conservation indications, thus, it is difficult to evaluate relationships between habitat features and turtle distribution based solely on these analyses. For example, no clear data exist on the relationships between *E. orbicularis* distribution and water quality or the availability of terrestrial habitat, despite the generally acknowledged importance of upland habitat for freshwater turtles (Naulleau, 1992; Semlitsch and Bodie, 2003).

In the absence of quantitative data on the habitat requirements of *E. orbicularis*, most conservation planners have used “expert judgement” to decide what areas are most suitable for reintroductions, or what management actions should be taken to improve habitat suitability for existing populations (e.g., Ballasina and Lopez-Nunes, 2000a; Schneider, 2000). However, a more objective approach based on quantitative data and statistical models can provide more accurate management indications (Lecis and Norris, 2003, and references therein). In turn, these indications could have value in optimising conservation efforts by concentrating funding on management areas and improving landscape features most important to the species.

We studied the distribution of *E. orbicularis* in the Po River delta, a large protected wetland system in northern Italy (Fig. 1). Our aim was two-fold. First, to assess the habitat requirement of the species, we evaluated habitat features related to the presence and abundance of *E. orbicularis* at the regional scale. We monitored turtle presence in a much greater number of biotopes than in previous studies, across more than 3000 ha of protected land. We measured environmental characteristics at the water, wetland, and landscape levels, since influences on turtle distribution could occur at all three of these levels. Our second aim was to integrate our data with the existing literature on the ecology and life history of pond turtles in order to evaluate possibilities for improving habitat for *in situ* conservation of threatened *E. orbicularis* populations, and habitat requirements to be met prior to turtle relocations.



**Fig. 1.** Study area; *a*: Punte Alberete-Valle Mandriole; *b*: Bosco della Mesola ; *c*: Campotto di Argenta; *d*: Fornace Violani reserve. Grey areas are woodlands.

## Material and methods

### *Study area*

Our study area was located in the Po River Delta Natural Park, one of the most important wetland systems in Italy. In past centuries, the pristine delta was largely reclaimed for agriculture and only a fraction of it remains unconverted. The most natural remnant wetlands are protected, distributed across an agricultural matrix. We surveyed 39 wetlands in four reserves (Fig. 1): Bosco della Mesola (1922 ha), Campotto di Argenta (1011 ha), Fornace Violani (3 ha) and Punte Alberete-Valle Mandriole reserve (480 ha). All four reserves are partially bordered by the agricultural landscape, and are composed of a complex mosaic of flooded broadleaf forest, mixed coniferous and broadleaf forest, ditches, ponds, swamps, and slow-flowing canals and rivers.

### *Turtle census*

We used visual transects and point counts to assess turtle presence in 39 wetlands (Heyer *et al.*, 1994; Quesada, 2000; Joyal *et al.*, 2001). Most censuses were performed by slowly driving a car (5 Km/h) around the border of the wetland. Census crews consisted of the driver and one or two observers searching for basking or swimming turtles. If bank morphology did not allow us to perform transects by car, two or three observers walked around the wetland to conduct the

survey. In large wetlands we also used a spotting scope (30x) to observe the furthest zones. If a wetland was >250 m across, we restricted our observations to fixed array point counts of 250 m, to reduce the risk of not detecting turtles. We performed transects by car because during preliminary trials we observed that turtles did not flee even though a car moved near them, possibly because they are accustomed to the passing of vehicles. We performed all transects on sunny days between 9 am and 3 pm, since during this period 40-60% of *E. orbicularis* present in wetlands are on basking sites (Cadi and Joly, 2000; Dall'Antonia *et al.*, 2001) and are easily detectable. We conducted census sessions in April, May, and early October (2002) since these are the periods of maximum activity for *E. orbicularis* (Lebboroni and Chelazzi, 1991). Each wetland was visited at least once during each census session, and most were visited twice, on different days.

### *Habitat features*

We recorded 17 environmental features for each wetland (Table 1). We collected a 0.5 litre water sample from each wetland in April for chemical and physical analysis.

Because freshwater turtles feed primarily on aquatic invertebrates and because invertebrate diversity is frequently used as an indicator of freshwater health, in May we evaluated macrobenthos richness within each wetland. Benthic macroinvertebrates were collected using a long-handled pond net dip netting submerged vegetation, banks, and the bottom for at least 3 min. If the bottom was covered mainly by sand or silt, we also collected 1 litre of sediment that was subsequently sieved using a series of metallic-mail sieves (mesh size: 2, 0.5 and 0.25 mm). We stored macrobenthos in 70% ethanol and identified them to the most specific taxon possible using a stereomicroscope.

We recorded morphology (surface, water depth and banks slope) and vegetation in the field during May; percentage data were approximated to the nearest 10%. Vegetation was recorded visually estimating the % cover in a 30 m wide strip surrounding the wetland. Sun exposure was recorded as the % of entire wetland surface exposed to direct sunlight, measured in June during sunny days between 11 am and 1 pm. We also recorded the presence of the North American slider turtle *Trachemys scripta elegans* (Wied-Neuwied, 1839), introduced in many European wetlands and a possible competitor of *E. orbicularis*. The percentage of surrounding woodlands in the nearest 250 m was evaluated in the field, and thus measured with a Geographic Information System (ArcView 3.2) on the basis of 1:25,000 maps and of CORINE land cover maps. We chose a radius of 250 m since 150-300 m is the proposed extent of terrestrial buffer zones for amphibians and reptiles (Semlitsch and Bodie, 2003). Moreover, we measured woodland cover also at a radius of 100, 500, 1000, 1500 and 2000 m, to evaluate the importance of forested habitat at different distances from the wetland.

Description	Code
Wetland surface (m <sup>2</sup> )	AREA*
Maximum water depth (cm)	WAT DEPTH*
Water permanence between February and October (y/n)	WAT PERM
Banks slope (mean of 4 measures on N, S, E, W, banks.)	BANKS
% of wetland covered by floating vegetation	FLOAT VEG
Presence of deadwoods or trunks emerging from the wetland surface (y/n)	TRUNK
Sun exposure % (see text)	SUN EXPOS
Surrounding grass % (see text)	GRASS%
Surrounding scrub % (see text)	SCRUB%
Surrounding tree % (see text)	TREE%
Wood cover % in the nearest 250 m (see text)	WOOD250
<i>Trachemys scripta</i> presence (y/n)	TRACHEM.
Nitrates concentration (mg/l)	NITRATES
Sulphates concentration (µg/l)	SULPHATES
Phosphates concentration (µg/l)	PHOSPHAT.
Water conductivity (µs)	CONDUCT.
Macrobenthos ( <i>n</i> of taxa)	MACROB.

\*: variable log transformed prior to analysis

**Tab. 1.** Environmental features measured.

#### *Assessment of census effectiveness*

Our census method was mainly based on observation of basking turtles. Basking behaviour could be influenced by time of day or by wetland features such as bank morphology, sun exposure, or presence of basking logs (Di Trani and Zuffi, 1997; Cadi and, Joly 2003). For example, it is possible that in sunny wetlands turtles bask earlier in the day, and therefore are active during the hottest hours, while in shaded wetlands turtles could also bask during the central hours of the day. Such behavioural differences between wetlands could lead to underestimation of turtle presence in sunny wetlands surveyed during the hottest hours, and should be detected by a significant interaction effect between time of survey and sun exposure on turtle observation. To determine if differences in wetland features resulted in differences in timing of basking behaviour, we tested whether interaction between habitat features and time of census caused differences in detectability of turtles. First, we subdivided the timing of census into three intervals (before 11 am, 11 am to 1 pm, after 1 pm) and considered timing as a factor (HOUR). Next, we calculated turtle abundance in each survey as (number of basking turtles)/(wetland area). Finally, we used a multi-factorial ANOVA to test the effect of month of sampling, hour, bank slope, sun exposure, trunk presence, and the interactions between month of sampling, hour and these three habitat features (Table 2) on the observed abundance of basking *E. orbicularis* at each survey. For this analysis, we only used data from surveys of the 22 wetlands occupied by *E. orbicularis* (see results).

#### *Relationship between turtle distribution and habitat*

We considered a wetland to be occupied by *E. orbicularis* if we observed at least one individual in one survey. Since pond turtle occurrence was not different between wetlands in the four reserves (Likelihood ratio:  $\chi^2 = 1.770$ ,  $df = 3$ ,  $P = 0.621$ ), all data were pooled for subsequent analysis. We used multiple logistic and linear regression models to relate *E. orbicularis* distribution to environmental variables, testing for multicollinearity using the correlation matrix between variables (Table S1): if  $|r| > 0.7$  the regression may be biased (Berry and Feldman, 1985). All correlations with  $|r| > 0.7$  involved four variables: sun exposure, grass%, tree% and woodland%. These variables were strongly correlated (Table S1: wetlands surrounded by woods had low grass cover, low sun exposure and high tree cover:  $|r| \geq 0.63$  in all pairwise correlations), thus they cannot be included together in the analysis. Therefore, prior to logistic and linear regression analyses, we performed a Principal Components Analysis (PCA) on these variables, and replaced them with the extracted factor(s). PCA extracted only one component (eigenvalue greater-than-one rule: eigenvalue = 3.215) explaining 80.37% of the variance of the four factors. The extracted factor was negatively correlated with sun exposure ( $r = -0.912$ ) and grass% ( $r = -0.894$ ) and positively correlated with tree% ( $r = 0.936$ ) and woodland% ( $r = 0.841$ ). We called this factor WOODENESS and added it to all the models as an environmental characteristic, in lieu of the four original factors. After replacement of the collinear variables with the factor WOODENESS, no pairwise correlations were  $\geq 0.7$ .

Logistic regression was used to relate presence/absence of *E. orbicularis* to environmental characteristics. A forward stepwise procedure was used to assess which variable should be added to the model: we used the likelihood ratio to select the variables that further reduced the log-likelihood of the model (Menard, 1995), until any new variable did not reduce it by any significant value. A variable was retained in the final model if remove  $p$  on the last step was  $< 0.05$ . We used Hosmer and Lemeshow  $r^2$  ( $R^2_L$ ) to evaluate the percentage of variance explained by our model (Menard, 1995). We inspected residual deviance in order to check for overdispersion. Since residual deviance (32.816) was not greater than the residual degrees of freedom (35), data were not overdispersed, suggesting that the error structure was appropriate for the data and the predictors were adequate to describe our data set (Rushton *et al.*, 2004) We calculated *E. orbicularis* abundance in each wetland as the ratio (maximum number of *E. orbicularis* seen in a wetland at one time)/(wetland area). We are aware that our estimate of *E. orbicularis* abundance could be inaccurate, since we did not validate it using capture-mark-recapture methods. However, it could be considered a reliable estimate of the relative abundance among wetlands. We performed a forward stepwise multiple linear regression on the subset of wetlands occupied by turtles, to determine if environmental characteristics influence turtle abundance among occupied wetlands.

We evaluated the performance of the logistic and linear multiple regression models using a jackknife procedure. Each wetland was removed from the dataset in turn, and the model of *E. orbicularis* distribution was evaluated with the remaining data. We calculated the predicted occupancy of the removed wetland using the resulting model parameters. We then compared predicted and observed distributions to evaluate the percentage of correct classification of wetlands (logistic regression model) and the relationship between predicted and observed *E. orbicularis* abundance (linear regression model).

Factor	<i>F</i>	df	<i>P</i>
MONTH	6.583	2	<b>0.002</b>
HOUR	0.677	2	0.511
MONT*HOUR	1.542	3	0.210
BANKS	0.928	1	0.338
SUN EXPOS	1.353	1	0.248
TRUNK	0.052	1	0.820
MONTH*BANKS	5.640	2	<b>0.005</b>
MONTH*SUN EXP	1.131	2	0.328
MONTH*TRUNK	2.417	1	0.124
HOUR*BANKS	0.295	2	0.745
HOUR*SUN EXP	0.383	2	0.683
HOUR*TRUNK	1.011	1	0.317
Error		84	

**Tab. 2.** Effects of survey month and hour, wetland features, and interactions between them, on observed *E. orbicularis* basking abundance (multi-factorial ANOVA). Significant results in **bold**.

To evaluate how much upland habitat is necessary for the presence of *E. orbicularis*, we repeated the logistic regression analysis by considering the wood cover at several distances from the wetland (100, 250, 500, 1000, 1500 and 2000 m). In turn, the wood cover at each of these distances was added to a logistic regression model including as independent variables wetland surface and water permanence (see results). The importance of wood cover at these six distance was evaluated as the likelihood ratio of the variable, a largest value of likelihood ratio suggesting that a variable is more strongly related to the turtle presence. In a similar fashion, the importance of each of these six variables for the abundance of *E. orbicularis* was evaluated by using the Pearson's correlation coefficient between the wood cover at a given distance and the turtle abundance.

We used Cook's distances to evaluate the presence of influential cases on the model. Since all Cook's distances were  $< 1$ , we assumed that no influential cases were present in our models (Cook and Weisberg, 1982). To meet assumptions of residual normality, *E. orbicularis* abundance, wetland area, and water depth were transformed using natural logarithms. All residuals were normally distributed after the transformations.

## Results

Twenty-two water bodies (56.4%) were occupied by *E. orbicularis* and 17 (43.6%) were unoccupied. We observed *E. orbicularis* in all four reserve areas. The highest observed abundance was 286 individuals / ha in a small canal (210 m<sup>2</sup>).

We found higher abundance of basking turtles in April and May than in October ( $F_{2,84} = 6.583$ ,  $P = 0.002$ ), but found no significant differences in basking turtle density related to sampling hour, sun exposure, basking log presence, or bank slope (Table 2). We found a significant interaction between bank slope and sampling month: we rarely observed *E. orbicularis* in wetlands with low bank slope in October ( $F_{2,84} = 5.640$ ,  $P = 0.005$ ). However, in October we never observed turtles in wetlands where they had not been previously observed, or at higher abundances than previously recorded. We found no significant interaction effects between sampling month and either sun exposure or basking log presence, or between sampling

Terms in model	<i>B</i>	$\chi^2$	Remove <i>P</i>	Model $\chi^2$	Model <i>P</i>	Model $R^2_L$	% correct
WAT PERM	+4.369 (4.043/6.356)	10.408	0.0013				
WOODEDNESS	+2.894 (2.700/4.055)	14.758	0.0001				
AREA	+0.795 (0.715/0.990)	8.418	0.0037				
Constant	-8.913 (-11.416/-8.414)			20.607 (df=3)	0.0001	0.386	74.4%

**Tab. 3.** Logistic regression model explaining *E. orbicularis* distribution. *B*: logistic regression coefficients; in parentheses: minimum and maximum *B* values estimated using the jackknife procedure. Correct classification % was calculated using a jackknife procedure. Degrees of freedom =1 unless specified.

hour and any habitat feature (Table 2). Therefore, we assumed basking behaviour was consistent across wetlands.

#### *Presence of T. scripta elegans*

We observed *T. scripta elegans* in only 6 of the 39 wetlands. They were associated with wetlands having large area (Likelihood ratio test:  $\chi^2 = 4.431$ ,  $df = 1$ ,  $P = 0.037$ ), rich macrobenthos communities ( $\chi^2 = 7.124$ ,  $df = 1$ ,  $P = 0.008$ ), and low WOODEDNESS ( $\chi^2 = 4.071$ ,  $df = 1$ ,  $P = 0.044$ ). No pairwise relationships between *T. scripta elegans* presence and the other environmental features were significant (all  $P > 0.1$ ). We also found no significant relationship between the presence of *T. scripta elegans* and *E. orbicularis* ( $\chi^2 = 0.118$ ,  $df = 1$ ,  $P = 0.732$ ).

#### *Water characteristics*

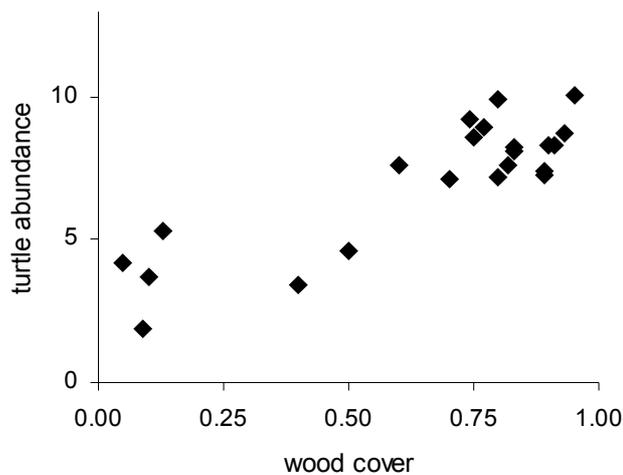
Wetlands occupied by *E. orbicularis* showed slightly lower mean conductivity than unoccupied wetlands ( $t_{37} = 0.218$ ,  $P = 0.036$ ). Univariate *t*-tests found no differences between occupied and unoccupied wetlands in nitrate, sulphate, or phosphate concentration, or in macrobenthos richness (all test:  $P > 0.5$ ). We observed *E. orbicularis* in wetlands with high concentrations of nitrates (range: 66.37-773.93 mg/l), sulphates (2.57-19.22  $\mu\text{g/l}$ ), and phosphates (26.55-201.56  $\mu\text{g/l}$ ), a wide range of conductivity (2.03-1388  $\mu\text{s}$ ), and in wetlands with very poor macrobenthos communities.

#### *Presence and abundance of E. orbicularis*

*Emys orbicularis* presence was related to water permanence, WOODEDNESS, and wetland area (Tab. 3). This model suggests that *E. orbicularis* prefers large, permanent wetlands surrounded by mature woods, and therefore having low sun exposure and minimal adjacent grass cover. All but two occupied wetlands had permanent water. No physical-chemical wetland characteristics were entered in the model. Using the jackknife procedure, the model correctly predicted *E. orbicularis* distribution in 74.4 % of the cases.

Variable	<i>B</i>	<i>t</i>	<i>p</i>
WOODEDNESS	+1.674 (1.593/1.762)	6.204	<0.0001
WAT PERM	+1.962 (1.823/2.112)	2.413	0.026
constant	+0.885 (0.790/0.973)		

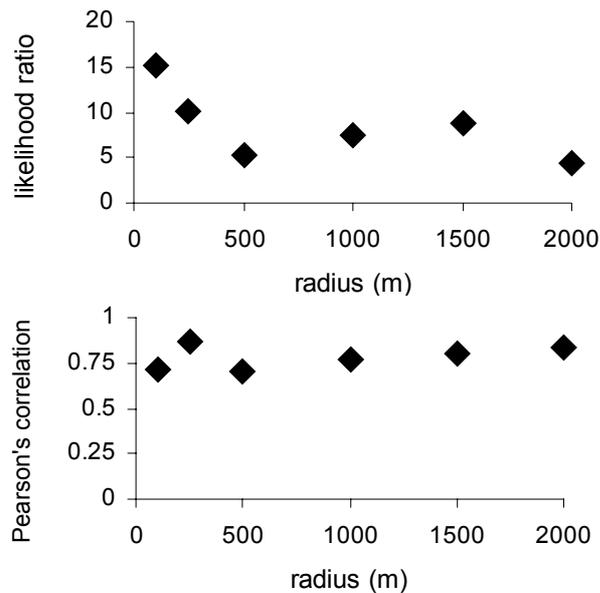
**Tab. 4.** Multiple regression model: effect of habitat features on *E. orbicularis* abundance. *B*: multiple regression coefficient; in parentheses: minimum and maximum *B* values estimated using the jackknife procedure.



**Fig. 2.** Relationship between wood cover at 250 m of distance from the wetland and turtle abundance (log transformed).

*E. orbicularis* abundance in occupied wetlands was positively related to water permanence and WOODEDNESS ( $F_{2,19} = 19.279$ ,  $P < 0.0001$ ,  $r^2 = 0.670$ ; see Table 4). *E. orbicularis* abundance predicted using the jackknife procedure was strongly related to observed abundance ( $F_{1,20} = 31.932$ ,  $P < 0.0001$ ,  $r^2 = 0.615$ ); coefficients calculated using the jackknife procedure were consistent across the overall dataset (Table 4). WOODEDNESS seems to be the most important variable in explaining *E. orbicularis* abundance. If used as the sole independent variable in a linear regression model, it explains 57% of variance ( $F_{1,20} = 26.378$ ,  $P < 0.0001$ ,  $r^2 = 0.569$ ). The highest abundances of *E. orbicularis* were observed in wetlands with more than 60% of wood cover in the nearest 250 m (Fig. 2).

The percentage of wood cover at all the six distance measured was positively related to the presence and the abundance of *E. orbicularis* (all  $P < 0.04$ ). Wood cover at small radius (100-250 m) was the most strongly related to the turtle presence (likelihood ratio = 15.146 and 10.101 respectively, see Fig. 3a). *Emys orbicularis* presence was strongly related also to the wood cover at a radius of 1500 m (likelihood ratio = 8.871). Wood cover at 250 m of radius was the most strongly related to turtle abundance, but strong relationships were observed also for a radius of



**Fig. 3.** Relationship between percentage of wood cover at six concentric distances from the wetlands and (a) presence and (b) abundance of *E. orbicularis*. Highest values of likelihood ratio and Pearson's correlation suggest that a variable is more strongly related to turtle distribution.

1000, 1500 and 2000 m (Fig. 3b). Anyway, all the six concentric measures of wood cover were strongly correlated to each other (in all pairwise correlations,  $r \geq 0.720$ ,  $P < 0.0001$ ).

## Discussion

Our results show that both wetland features and features of the habitat surrounding wetlands are important for *E. orbicularis* presence. Interestingly, the two habitat features most important for *E. orbicularis* presence in the 39-wetland dataset (water permanence and WOODEDNESS) were also the most important for abundance of the species among the subset of 22 occupied wetlands. Therefore, we conclude that these two variables should be considered crucial habitat characteristics for *E. orbicularis* in habitat management actions aimed at protection of this species. We are aware that our study dealt with only one geographic area and a limited sample of wetlands. However, because the existing literature supports our findings (see below), we believe our results could be useful for conservation planning across the entire range of *E. orbicularis*. Moreover, our conclusions are strengthened by the observations that the factors related to species presence are also related to species abundance, no cases strongly influence the models, and all jackknife models yielded very similar results (Tables 3 and 4). Therefore, we assume that our model results are at least partially applicable to other landscapes for assessing *E. orbicularis* conservation plans (see Whittingham *et al.*, 2003). Further research in other landscapes could lead to better understanding of *E. orbicularis* ecology across its range.

*Habitat requirements of E. orbicularis*

Water permanence is an important habitat feature for many freshwater turtle species (Bodie *et al.*, 2000); our study confirms its importance for *E. orbicularis* (Tables 3; 4). The variable WOODENESS, a factor positively related to percentages of surrounding woodlands and riparian trees, and negatively related to percentages of surrounding grass and wetland sun exposure, has great importance for *E. orbicularis* presence and abundance. This factor mainly depends upon the terrestrial habitat surrounding the wetland, since water bodies surrounded by woodlands are intrinsically shaded. Pond turtles frequently use upland habitats for many activities (Semlitsch and Bodie, 2003). Female turtles sometimes move long distances for egg laying, and can choose nesting sites several hundred meters from their residential ponds, often in open areas near woodlands (Rovero and Chelazzi, 1996; Jablonski and Jablonska, 1998; Schneeweiss, and Steinhauer, 1998; Andreas, 2000; Meeske, 2000; Utzeri and Serra, 2001), likely because the choice of nesting site at both micro- and macro habitat levels can be critical for turtle breeding success (Spencer and Thompson, 2003). Woodlands surrounding a wetland allow turtles to move relatively long distances and to find suitable nesting sites. The presence of woodlands reduces sun exposure during migration, therefore reducing the risk of dehydration, and provide a more open understory which allows easier movement of turtles. A wooded landscape could also be favourable for hatchlings when they move from nest to wetland. Moreover, terrestrial habitat and leaf litter provided by woodlands can be used during aestivation and hibernation (Naulleau, 1992; Fritz and Gunther, 1996; Utzeri and Serra, 2001).

Woodlands are frequently the preferred upland habitat for terrestrial movements (Naulleau, 1992). Therefore, we hypothesize that a more natural, wooded terrestrial landscape increases the connectivity of the matrix between wetlands, allows greater movement of individuals among populations and the existence of a metapopulation, and therefore enhances the likelihood of long-term *E. orbicularis* persistence in the landscape (Bennet, 1999; Joly *et al.*, 2001, Ficetola and De Bernardi, 2004). Finally, the environment surrounding wetlands strongly influences the aquatic habitat. For example, the presence of trunks and dead wood in wetlands is positively associated with WOODENESS (Likelihood ratio:  $\chi^2 = 22.473$ ,  $df = 1$ ;  $P < 0.0001$ ), and is very important for turtles. Trunk presence favours sub aerial basking (Cady and Joly, 2003), and dead wood in the water can be used as shelter or as a source of prey (Meeske, 2000).

We observed *E. orbicularis* in wetlands with high concentrations of nitrates and phosphates, and with poor macro benthos communities, indicating that this species can also live in eutrophic water. Studies of other freshwater turtle species have shown that they also can survive in water with high levels of organic pollution (Souza and Abe, 2000). Therefore, water eutrophication does not appear to be limiting factor for the presence of *E. orbicularis*. However, it is possible that chemical pollution contributed to the decline of this species in disturbed landscapes; further studies are required to test this hypothesis. The absence of *E. orbicularis* from the wetlands with highest conductivity ( $>1400 \mu\text{s}$ ) is likely caused by the presence of brackish water in these wetlands, which are near the coast.

## *Management implications*

By integrating our results with data from the literature, we are able to provide several suggestions for *E. orbicularis* habitat management plans. First of all, it is important for the persistence of this species to preserve large areas of natural habitat, including both wetland and terrestrial habitat. Scientists and conservation planners have frequently overlooked the importance of upland habitat for semi-aquatic vertebrates (Gibbons, 2003). Several recent studies outline the pivotal role of the terrestrial environment, not only for protection of water resources and aquatic ecosystems (Kiffney *et al.*, 2003, and references therein), but also to permit several critical life-history functions of semi-aquatic species, allowing long-term survival of populations (Burke and Gibbons, 1995; Nolet and Rosell, 1998; Joyal *et al.*, 2001; Gibbons, 2003; Semlitsch and Bodie, 2003; Schabetsberger *et al.*, 2004). 250-300 m has been reported as the buffer zone encompassing most terrestrial activity for many freshwater turtles (Burke and Gibbons, 1995; Semlitsch and Bodie, 2003). However, we showed that *E. orbicularis* presence and abundance is strongly related also to wood cover at wider radii. Our data do not allow to unambiguously evaluate how much terrestrial habitat is enough for *E. orbicularis*, since all the six measures of wood cover are strongly intercorrelated and not independent. Anyway, *E. orbicularis* frequently moves 1000-2000 m or more from wetlands during terrestrial activities (e.g., over 4 km reported by Jablonski and Jablonska, 1998), distances much greater than those recorded for many other freshwater turtles (see data on 28 turtle species reported by Semlitsch and Bodie, 2003). Thus, the extent of terrestrial buffer zones proposed for other turtles species may not be adequate for *E. orbicularis*: we suggest protecting a terrestrial area extending at least 1000 – 1500 m from wetlands. This area should ideally include open areas with soft soil and good sun exposure (i.e., south-facing slopes) for nesting (Rovero and Chelazzi, 1996; Schneeweiss *et al.*, 1998; Andreas, 2000; Ballasina and Lopez-Nunes, 2000b; Chelazzi *et al.*, 2000).

Moreover, turtles in different life history stages may utilize different types of wetlands. For example, hatchlings require shallower wetlands than adults do, to avoid the risk of drowning; temporary wetlands may be used for hibernation; ditches and ponds can be used during migrations as stepping stones or to move across the landscape (Rovero and Chelazzi, 1996; Schneeweiss and Steinhauer, 1998; Andreas, 2000; Rossler, 2000; Utzeri and Serra, 2001). Therefore, the presence of a complex system of water bodies as well as upland habitat should be protected for the persistence of *E. orbicularis*, with some large, permanent wetlands for the aquatic life of adults, and smaller and/or semi-permanent wetlands for other life history stages. The presence of multiple wetlands could also facilitate the existence of metapopulations, increasing the likelihood of long-term survival of populations (Hanski and Gilpin, 1997).

At the wetland level, one useful and relatively inexpensive habitat improvement for freshwater turtles is to provide basking logs, such as trunks and dead woods (Spinks *et al.*, 2003). Because the presence of mature woodlands in uplands surrounding wetlands naturally results in the fall of wood into wetlands, thus a long-term wetland management perspective should prioritise upland habitat preservation.

We did not analyse the effects of shoreline vegetation because in almost all the analysed wetlands it was quite abundant, covering 75-100% of the banks. However, several studies describe the importance of shoreline vegetation to *E. orbicularis*: it provides shelter, abundant

food items, and easier access to upland habitat than allowed by steeply-sloped, naked banks (Lebboroni and Chelazzi, 1991; Andreas, 2000; Meeske, 2000). Therefore, another important goal for improved turtle habitat suitability is the presence of natural banks with abundant vegetation.

*Trachemys scripta elegans* was not abundant in our study area, probably because human exploitation of this area remains low. Therefore, *T. s. elegans* is limited to wetlands surrounded by the most human-modified landscapes (i.e. areas with low WOODENESS), and we did not observe a negative effect of the presence of *T. s. elegans* on *E. orbicularis*. However, in many areas of Europe, *T. s. elegans* is now much more common than *E. orbicularis*, and this introduced species can successfully compete with *E. orbicularis* from basking sites and other resources (Arvy and Servan, 1998; Cadi and Joly, 2003). The problem of competition between *E. orbicularis* and *T. s. elegans* could become more severe in the future, because new individuals of *T. s. elegans* continue to be released in natural wetlands, and because some populations of introduced *T. s. elegans* successfully breed in southern Europe. Therefore, *T. s. elegans* should be promptly removed from all wetlands, to avoid further cases of acclimatisation and competition with the native fauna (Martinez-Silvestre *et al.*, 1997; Quesada, 2000; Cadi and Joly, 2003; Ficetola *et al.*, 2003).

Our study suggests that habitat management plans for *E. orbicularis* conservation should protect many features of wetlands and the landscapes surrounding them. The habitat requirements of this species are not easily satisfied in the human-dominated lowlands of Europe that were once pristine *E. orbicularis* habitat. In particular, this species requires relatively large wetlands and/or wetland systems for different life history stages, and quite large natural, wooded terrestrial habitats to allow inter-wetland movements, reproduction, and other terrestrial activity. Therefore, pond turtle conservation plans should focus not only on wetland features, but also on features of upland habitat surrounding wetlands. Planning should take a broad “landscape level approach” (Joyal *et al.*, 2001) to allow population persistence, and also a long-term approach, since these long-lived animals have a long generation time and require many years for demographic growth.

**Table s1.** Product-moment correlations (Spearman's  $r$ ) between habitat features. In **bold**,  $|r| > 0.7$ .  $N = 39$  for all pairs. Abbreviations in Table 1.

	W depth	W perm	Banks	Float veg	Trunk	Sun exp	Grass%	Scrub%	Tree%	Wood250	Trachem.	Nitrates	Sulphates	Phospat.	Conduct.	Macro.
Area	0.378	0.509	0.276	0.023	-0.674	0.661	0.635	-0.108	-0.517	-0.667	0.334	0.154	0.374	-0.117	0.072	-0.039
Wat depth	1	0.554	0.277	0.217	-0.261	0.319	0.209	-0.164	-0.186	-0.284	0.144	0.197	0.315	0.337	0.014	0.111
Wat perm		1	0.319	0.121	-0.256	0.476	0.542	0.252	-0.321	-0.360	0.234	-0.128	0.076	-0.020	-0.173	0.045
Banks			1	-0.106	-0.058	0.289	0.165	-0.068	-0.190	-0.247	0.280	0.190	0.341	0.037	-0.298	0.210
Float veg				1	0.069	-0.108	-0.261	-0.156	0.257	0.152	-0.206	-0.135	0.002	0.232	-0.285	-0.259
Trunk					1	-0.579	-0.496	0.170	0.613	0.625	0.014	0.026	-0.221	-0.050	-0.040	-0.111
Sun exp						1	<b>0.810</b>	-0.177	<b>-0.815</b>	-0.633	0.206	0.004	0.389	-0.133	0.227	0.005
Grass%							1	0.113	<b>-0.761</b>	-0.630	0.295	-0.032	0.134	-0.173	0.259	0.038
Scrub%								1	0.090	-0.092	0.037	-0.120	-0.231	-0.160	0.114	0.142
Tree%									1	<b>0.772</b>	-0.214	-0.027	-0.240	0.057	-0.300	-0.173
Wood250m										1	-0.445	-0.102	-0.336	-0.117	-0.339	-0.177
Trachemys											1	0.164	0.190	0.200	0.104	0.425
Nitrates												1	0.321	-0.076	0.193	0.137
Sulphates													1	0.274	-0.104	-0.004
Phosphates														1	-0.192	0.141
Conductibility															1	0.014
Macrobenthos																1

#### 4. AMPHIBIANS IN A HUMAN-DOMINATED LANDSCAPE: THE COMMUNITY STRUCTURE IS RELATED TO HABITAT FEATURES AND ISOLATION

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*Biological Conservation*, 119: 219-230

##### **Abstract**

We studied amphibian populations in a human-dominated landscape, in Northern Italy, to evaluate the effects of patch quality and isolation on each species distribution and community structure. We used logistic and linear multiple regression to relate amphibian presence during the breeding season in 84 wetlands to wetland features and isolation. Jackknife procedure was used to evaluate predictive capability of the models. Again, we tested the response of each species to habitat features related to the richest communities. Amphibian presence depends strongly on habitat quality and isolation: the richest communities live in fish-free, sunny wetlands near to occupied wetlands. The negative effects of isolation do not seem to be biased by spatial autocorrelation of habitat features. The system shows strong nestedness: amphibian persistence depends on the contemporary effects of species adaptability and mobility. The commonest species, the pool frog (*Rana synklepton esculenta*) and the Italian tree frog (*Hyla intermedia*), are able to move through the matrix using canals and hedgerows, and can maintain metapopulations across the landscape; the rarest species (newts and toads) are more sensitive to habitat alteration, and they are strongly affected by isolation effects. If human exploitation of the landscape continues, only few species, mobile and opportunistic, will persist in this landscape.

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##### **Introduction**

The expansion of land use subsequent to human population growth results in the fragmentation of natural landscapes (Wilcox and Murphy, 1985). Therefore, in the human-dominated landscapes, natural habitats are reduced to discrete patches, surrounded by a matrix exploited by agriculture or urbanization. Selective extinction and colonisation are two processes that forge community structure in patchy landscapes. Selective extinction causes the disappearance of some species from a habitat patch through different processes: a species may require a larger area for persistence and be incapable of using more than one patch (Schadt *et al.*, 2002), habitat alteration due to human activities may make the patch environment unsuitable (Telleria and Santos, 1994; Marsh and Pearman, 1997), or a decreased immigration rate may reduce the probability of a rescue effect (Hanski *et al.*, 1995). Moreover, genetic drift and inbreeding resulting from isolation may reduce fitness (Saccheri *et al.*, 1998; Rowe *et al.*, 1999). Also, stochastic processes may result in the disappearance of a population from a patch (Sjorgen, 1991; Ficetola and Scali,

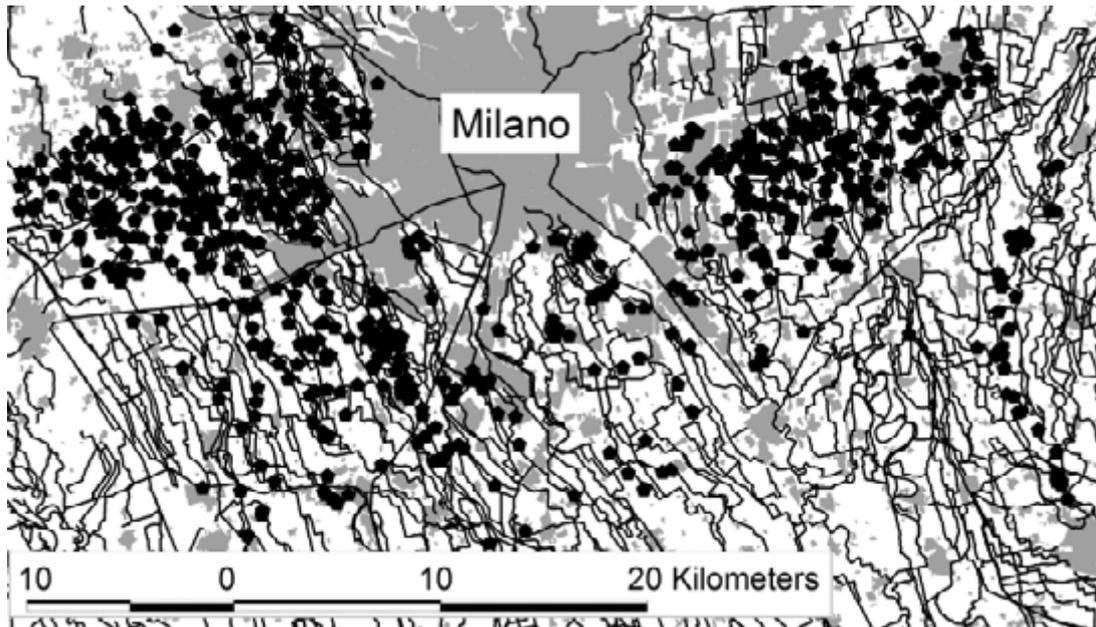
2002).

Successful colonisation of patches within a fragmented landscape is generally shown by the more mobile species living in fragments, able to cross the matrix (Sarre *et al.*, 1995; Johst *et al.*, 2002; Pires, *et al.*, 2002), by species living in the matrix between patches and having high resilience to human alterations of ecosystems (Harrison *et al.*, 2001) and by opportunistic species that are favoured by environmental alterations subsequent to human activities (Henein *et al.*, 1998).

Community systems where selective extinction and/or colonisation are active may show a nested pattern (e.g. Wright *et al.* 1998). A community set shows nestedness if the species composition of a patch that is poorer in species number is a proper subset of the richest ones (Patterson and Atmar, 1986). In this case, poorer patches have similar communities, composed of the most resistant or best coloniser species (Telleria and Santos, 1994; Hecnar *et al.*, 2002) and the conservation of poorer communities will not allow the biodiversity conservation in the landscape (Davidar *et al.*, 2002). Nested patterns can be related to ecological features of species or habitats, allowing the formulation of hypothesis about the extinction and colonization processes active in the landscape (Patterson *et al.*, 1996; Davidar *et al.*, 2002; Hecnar *et al.*, 2002).

Selective extinction and colonization can not be considered independently in metapopulation theory (Hanski and Gilpin, 1997). For example, in a source-sink dynamic, species that are good colonisers can reach new suitable patches and recolonise fragments where they previously became extinct (den Boer, 1990). Moreover, species able to survive with large populations in each patch will also avoid genetic drift in the presence of a low number of migrants (Russell, 1996). The situation for species that are less mobile, or highly dependent on key habitat features, is more critical. Therefore, determination of environmental features that allow the survival of species, their dispersion pattern, and interactions between survival and dispersion can help to explain extinction/colonization dynamics. The results of such studies are useful for landscape planning directed to the conservation of biodiversity.

The investigation of amphibian responses to environmental alteration is an important issue in studies of biological conservation because of the global amphibian decline (Barinaga, 1990; Houlahan *et al.*, 2000). Moreover, many amphibians have low mobility and strongly select habitat features. Thus they provide a useful test case with which study the effects of isolation and fragmentation on animal communities. Despite many studies about amphibian metapopulations considered each wetland a patch with its related population, this approach was recently criticised because many of the amphibian species that use the wetlands do so only in the reproductive season (Marsh and Trenham, 2001). Several studies have shown evidence of the importance of terrestrial habitats and landscape structure on these vertebrates (Vos and Stumpel, 1995; Vos and Chardon, 1998), suggesting that a larger scale approach could better explain the observed pattern (Marsh and Trenham, 2001).



**Fig. 1.** Study area (Lombardy region, Northern Italy). The study area surrounds the city of Milan to the west, south and east. Grey: urban areas; black line: canals and ditches; pentagons: springs and wetlands.

Here we present the results of the response of amphibian communities to habitat features in the lowland surrounding the city of Milan, in Lombardy (Northern Italy). On the global scale, lowlands are some of the most human-dominated landscapes, and Lombardy lowland is one of the European areas with the largest agricultural and industrial development (Provincia di Milano, 2003). However, in this area there still exists a complex hydrographic network and some patch with relatively low disturbance, where some populations of endangered amphibians still survive. The aims of this study are to find the ecological requirements that allow amphibian presence in wetlands, to evaluate the effects of isolation on population distribution, and to examine how isolation and habitat quality interact with the species natural history. Our results shed light on regional forces that drive species abundance and community structure.

### **Study area**

We investigated a surface area of 520 km<sup>2</sup> in the river Po floodplain (Lombardy region, Northern Italy), (Fig. 1). This area surrounds the city of Milan to the west, south and east and is comprised of the “Agricolo Sud Milano” and “Adda Sud” Regional Parks. The landscape is highly exploited by humans, dominated by the presence of urban suburbs and agriculture. Only a few small wooded fragments still exist and the wooded surface is less than 5 % of the landscape. The altitude is 60-160m and the geological features of this area cause the presence of numerous water springs used in the past for agriculture (Padoa-Schioppa, 2002). Agricultural irrigation of the region also causes the presence of thousands of channels that cross all the lowland. The web of hedgerows, traditionally used as boundaries between fields, covers 1.6% of the landscape’s surface (Baietto *et al.*, 2002). In the late 19th - early 20th century, 9 amphibian species were living in this area: *Triturus vulgaris*, *Triturus carnifex*, *Pelobates fuscus insubricus*, *Bufo bufo*,

*Bufo viridis*, *Hyla intermedia*, *Rana dalmatina*, *Rana latastei* and *Rana synklepton esculenta*. All of these, with only the exception of *T. vulgaris*, were very abundant near Milan (Campeggi, 1883; Vandoni, 1914).

## Methods

We detected amphibian presence in 84 wetlands (ponds, temporary pools and ditches). The surface area of analysed wetlands is highly variable, ranging between 6 and 90000 m<sup>2</sup> (median = 360 m<sup>2</sup>; mean = 4861 m<sup>2</sup>). We surveyed each wetland after dusk at least once every 3 weeks, during late winter, spring and early summer (February through June). The presence of calling males was recorded in 5-minute point counts, following a 1-minute pause between arrival and commencement of the survey. After the point count, we searched the entire perimeter of the wetland walking along the banks. We used a head-lamp to light up the wetland, recording all the adults, tadpoles or metamorphs seen. We also recorded the presence of egg masses of toads, Italian tree frog and Italian agile frog. Moreover, we deep-netted each wetland for tadpoles in May, sampling banks and every bottom type.

For each wetland, we recorded 15 environmental features (Table 1A) and 5 isolation features (Table 1B). We recorded morphology, soil and vegetation in the field during May. Surrounding vegetation was recorded as the % cover of a 30m strip surrounding the wetland. Sun exposure was recorded as the % of wetland surface exposed to the direct sunlight. We measured sun exposure in May during sunny days, between 11.00 am and 1.00 pm (UTM). Animal presence was recorded during each survey and if fish or ducks were detected at least once, we recorded them as present. We recorded isolation features from the map (1:10000 technical regional cartography), using a Geographic Information System. We used these 20 variables in multiple regression and in every logistic regression. Moreover, in each single species logistic regression we added 3 species-specific isolation features: distance from the nearest wetland occupied by the analysed species and the number of other occupied wetlands in the nearest 250 m and 1500 m (Table 1C).

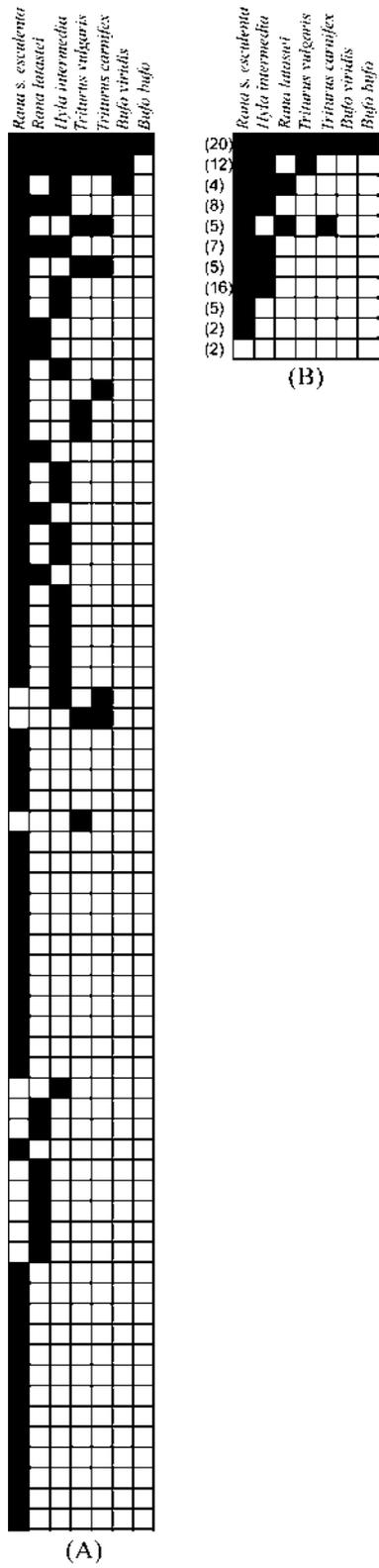
Since the “pond as patch” view of amphibian spatial dynamics may be an oversimplification (Marsh and Trenham, 2001), we repeated some analyses on two different spatial scales. We considered each wetland a single small-scale patch, and we clustered them in large scale patches. Large-scale patches are wetland groups without the presence of barriers (main routes, fences) between wetlands; in none of the large-scale patches the inter-wetland distance was higher than 3000 m, therefore we assumed that the more mobile amphibians species, like toads and pool frogs, are able to move across the overall patch (Sinsch, 1990; Tunner, 1992). All the large-scale patches are fully surrounded by routes and human exploited matrix (agricultural field and suburbs). We considered 11 large-scale patches; each large-scale patch included 2 to 16 wetlands (Fig. 2).

A. Environmental Features	Code	Spatial autocorrelation (Moran I)
Wetland surface (m <sup>2</sup> )	AREA <sup>a</sup>	0.134
Maximum water depth (cm)	WAT DEPTH <sup>a</sup>	0.163
Banks slope (°, mean of 4 measures on N, S, E, W banks)	B SLOPE	0.171
Water permanence between February and October (y/n)	WAT PERM	0.106
Sun exposure % (see text)	SUN EXPOS	-0.025
Soil granulometry (clay = 1, sand = 2, gravel = 3)	SOIL	0.344*
Submerged deadwood abundance (n/m <sup>2</sup> , along slopes)	SUB WOOD <sup>a</sup>	-0.010
% of wetland covered by submerged vegetation	SUB VEG	0.170
% of wetland surface covered by floating vegetation	FLOAT VEG	0.138
% of riparian vegetation along the shoreline	RIP VEG	0.018
Surrounding grass % (see text)	GRASS	0.513*
Surrounding scrub % (see text)	SCRUB	0.367*
Surrounding wood % (see text)	WOOD	0.199*
Fish presence (y/n)	FISH PRES	0.144
Ducks presence (y/n)	DUCKS PRES	0.053
<b>B. Isolation features used for every species</b>		
Distance from the nearest wetland (m)	DIST WET	
Number of wetland on the nearest 250 m	N 250	
Number of wetlands on the nearest 1500 m	N 1500	
Total number of species on the wetlands nearest than 250 m	SP 250 <sup>a</sup>	
Total number of species on the wetlands nearest than 1500 m	SP 1500	
<b>C. Species-specific isolation features</b>		
Distance from the nearest wetland occupied by the analysed species (m)	DIST SP <sup>b</sup>	
Number of wetland on the nearest 250 m occupied by the analysed species	SP <sup>b</sup> 250	
Number of wetland on the nearest 1500 m occupied by the analysed species	SP <sup>b</sup> 1500	

**Tab. 1.** Environmental and isolation features measured, and spatial autocorrelation of the environmental features. Expected Moran I =  $-0.012 \pm 0.071$ .

### *Statistical methods*

We evaluated if the habitat features recorded were spatially autocorrelated using the Moran I statistic; this analysis was repeated for each variable using the CrymeStat®II software package (Ned Levin and Associates, Houston, Texas). Since multiple tests were performed, we adjusted the significance level ( $\alpha$ ) of relationships for the number of tests performed (k) using the Bonferroni's method ( $\alpha' = \alpha/k$ ) (Sokal and Rohlf, 1995).



**Fig. 2.** Maximally nested matrix of species distribution. Each column represent the distribution of a species; each row represent a wetland (A, small scale patch), or a large scale patch (B). Between parentheses the number of wetlands per patch.bro

<i>T. carnifex</i>	Tc 250	Tc 1500
<i>DIST Tc</i>	-0.848	-0.890
<i>Tc 250</i>		0.664
<i>T. vulgaris</i>	Tv 250	Tv 1500
<i>DIST Tv</i>	-0.375	-0.666
<i>Tv 250</i>		0.671
<i>H. intermedia</i>	Hi 250	Hi 1500
<i>DIST Hi</i>	-0.373	-0.578
<i>Hi 250</i>		0.685
<i>R. latastei</i>	RL 250	RL 1500
<i>DIST RL</i>	-0.765	-0.789
<i>RL 250</i>		0.761
<i>R. s. esculenta</i>	RE 250	RE 1500
<i>DIST RE</i>	-0.469	-0.207
<i>RE 250</i>		0.728

**Table 2.** Pearson product-moment correlations among species-specific isolation features. N = 84 for all correlations. Abbreviations are in Table 1.

We used logistic regression to relate species presence or absence to environmental and isolation features. A forward stepwise procedure was used to assess which variable should be added to the model: we used the likelihood ratio to select the variables that further reduced the log-likelihood of the model, until any new variable did not reduce it by any significant value. To distinguish the environmental from isolation features effect, we used a blockwise procedure. We first added the environmental variables and the first block of stepwise logistic regression ended when no other environmental variable explained a significant portion of the log-likelihood. Afterwards, we added the isolation variables explaining a significant portion of residual log-likelihood. This approach allow to test if isolation parameters are additional explanatory factors to the habitat quality factors (Laan and Verboom, 1992; Vos and Stumpel, 1995). Since we analysed the relationship between the isolation features and the residuals of the relationship between species presence and habitat, we measured the effects of isolation on the variance unexplained by habitat features. Therefore, the isolation effects observed can be considered independent by the habitat features. We added the habitat features in the first block, and the isolation features in the second block, to avoid increasing the likelihood of adding an “isolation factor” into our model. Because of the Wald’s statistic use to assess the single variable significance may be unreliable (Menard, 1995), a variable was retained in the final model if remove  $P$  on the last step of each block were  $<0.05$ . We used Hosmer and Lemeshow  $R^2$  ( $R^2_L$ ) to estimate the proportional reduction in the absolute value of the log-likelihood measure. This measure is the proportion  $G_m/D_0$ , were  $G_m$  is the log-likelihood explained by the model and  $D_0$  is the initial log-likelihood:  $R^2_L$  is a good analogue of the  $R^2$  of linear regression for logistic regression (Menard, 1995). Similarly, we used multiple stepwise linear regression to relate the species richness of each community to the environmental and isolation features. In the first block we added the environmental variables explaining a significant portion of variance; in the second one the isolation variables.

In the logistic and linear multiple regression models, we tested for multicollinearity using the correlation matrix between variables: if  $r > 0.7$ , then the regression may be biased (Berry and Feldman, 1985). We did not find multicollinearity between the habitat features or between the isolation features used for every species (data not shown: in all pairwise correlations  $r < 0.7$ ).

However, we detected strong correlation between species-specific isolation variables in *T. carnifex*, *R. latastei* and *R. esculenta* (Table 2). Since the intercorrelated variables are descriptors of the same factor, for these three species we used in the models only the variables with the higher partial correlation with the species presence (DIST TC, RL 1500, DIST RE and RE 250) (Bowerman and O'Connell, 1990). The inclusion of any of the excluded variables *in lieu* of the correlated variables would not change any model (data not shown).

We tested the effect of environmental variables selected by multiple regression on each species, to test if each species response show the same pattern of the full community, using one-tailed Fisher exact test and t-test. We used G test (after William's correction) to check for association between WAT PER and FISH and Mann-Witney U test for univariate association between environmental features and species richness. If necessary, we log-transformed variables, to better meet the assumption of residual normality in multiple logistic and linear regression (Sokal and Rohlf, 1995).

In order to examine the degree of nestedness we used the method proposed by Atmar and Patterson (1993). We used the  $T^\circ$ -value to evaluate the degree of nestedness.  $T^\circ$  may vary between  $0^\circ$  and  $100^\circ$ , and it is an absolute disorder index, based on the differences between a theoretical perfectly nested set ( $T^\circ=0^\circ$ ) and the observed system (Atmar and Patterson, 1993). The significance of  $T^\circ$  is assessed using Monte Carlo simulation. The null-model used by Atmar and Patterson Nested Calculator (1993; 1995) to evaluate the nestedness were recently criticised, since it can lead to overestimate the nestedness level (Fisher and Lindenmayer, 2002). Therefore, we repeated nestedness analysis using two null-models. The Atmar and Patterson null-model (thereafter *null-model 0*) assumes that each cell of presence/absence matrix has an equal probability of being occupied: it does not differentiate between species or patches, and only the total number of occupied cells in each simulated matrix is constant. The Fisher and Lindenmayer (2002) null-model (thereafter *null-model 1*) assumes that some species are more common in species assemblages, and cells in random matrices are occupied on the basis of the probability of encountering a given species in the landscape. The use of *null model 2* yields a more conservative inference about the significance of nestedness. Our statistic was  $P$ , the probability of a random replicate being equally or more nested than the observed matrix. We generated random matrices based on *null-model 0*, and we calculated the related  $P$  using the program Nested Calculator (Atmar and Patterson, 1995); we generated random matrices based on *null-model 1* using the program Random Matrix Generator (Colombo, 2003), and subsequently we loaded them into the Nested Calculator. The  $T^\circ$  values for each matrix were recorded; then we calculated the mean and standard deviation of the resulting distribution of  $T^\circ$  values. We calculated the associated  $P$  values using a two tailed z test. For each null-model we performed 100 runs of Monte Carlo simulation. To detect nested patterns on different spatial scales, we repeated the analysis considering each wetland and each large-scale patch as a sampling unit.

Species	Occurrence		Protection status <sup>d</sup>
	a	b	
<i>Triturus carnifex</i>	7	2	2, 4
<i>Triturus vulgaris</i>	8	3	/
<i>Bufo bufo</i>	1	1	/
<i>Bufo viridis</i>	3	1	4
<i>Hyla intermedia</i>	19	7	4
<i>Rana latastei</i>	16	2	2, 4
<i>Rana s. esculenta</i>	56	10	5
<i>n</i> wetlands/patches	84	11	

a: occurrence in wetlands

b: occurrence in large-scale patches

c: Enclosures of Habitat Directive (CEE 92/43) were the species is listed. 2: species requiring the designation of special conservation areas; 4: species requiring rigorous protection; 5: species that could become object of management measures.

**Tab. 3.** Species occurrence in the study area, and their protection status

## Results

Data about soil granulometry and about vegetation surrounding the wetlands (grass, scrubs and wood) are spatially autocorrelated (Table 1): close wetlands have similar values for these four variables; spatial autocorrelation is lacking or weak for the remaining variables. We found seven amphibian species in the study area: two urodelans (Italian crested newt *Triturus carnifex* and smooth newt *Triturus vulgaris*) and five anurans (common toad *Bufo bufo*, green toad *Bufo viridis*, Italian tree frog *Hyla intermedia*, Italian agile frog *Rana latastei* and pool frog *Rana synklepton esculenta*). The pool frog and Italian tree frog are the commonest species; the occurrence of each species in single wetlands and large-scale patches is shown in Table 3. Five species are protected because they are listed in the Habitat Directive of European Union (CEE 92/43; Table 3).

### *Species presence*

Since we found the common toad and the green toad in only one and three ponds respectively, it was not possible to perform logistic regression models for these two species; we built models for all the other species. Water depth, water permanence, sun exposure and fish presence seem the most important environmental features determining amphibian presence; we did not find any significant effects of vegetation. Environmental features and isolation features too are good predictors for presence in each species model; moreover, only the Italian tree frog presence seems not to depend on isolation from conspecific populations. For each species, environmental features explain 14-35% of total variance; isolation alone explain 15-25% of residual variance. The performance of the models was evaluated using a jackknife procedure. In turn, each of the 84 wetlands was removed from the dataset and the models of species occupancy were evaluated

Specie	Terms in model	Effect sign	Enter $\chi^2$	Enter $P$	Remove $P$	Block $P$	Block $R^2_L$	Model p	Model $R^2_L$	% correct
<i>T. carnifex</i>	WAT PERM	-	9.994	<0.001	<0.0001					
	WAT DEPTH	+	7.081	<0.01	<0.01	<0.0001 (df=2)	0.354			
	DIST Tc	-	11.035	<0.001	<0.001			<0.0001 (df=3)	0.583	94%
<i>T. vulgaris</i>	FISH PRES	-	7.211	<0.01	<0.01	<0.01	0.138			
	Tv 250M	+	9.082	<0.001	<0.001			<0.001 (df=2)	0.311	94%
<i>H. intermedia</i>	SUN EXPOS	+	28.988	<0.0001	<0.0001					
	FISH PRES	-	5.618	<0.05	<0.05	<0.0001 (df=2)	0.390			
	SP 250M	+	12.828	<0.001	<0.001			<0.0001 (df=3)	0.534	90%
<i>R. latastei</i>	SUB WOOD	+	13.468	<0.0001	<0.001					
	WAT DEPTH	+	5.051	<0.05	<0.05	<0.0001 (df=2)	0.226			
	RI 1500M	+	21.604	<0.0001	<0.0001			<0.0001 (df=3)	0.490	87%
<i>R. s. esculenta</i>	SUN EXPOS	+	17.637	<0.0001	<0.0001	<0.0001	0.165			
	Dist Re	-	17.589	<0.0001	<0.0001			<0.0001 (df=2)	0.329	79%

**Tab. 4.** Logistic regression models for species presence. Correct classification % was calculated using a jackknife procedure.

with the remaining data. Using the resulting model parameters, we calculated the predicted wetland occupancy of the removed wetland for each species. Therefore, we compared the predicted occupancy value with the observed one (e.g. Ciucci *et al.*, 2003). This procedure was repeated for each of the 84 wetlands and for each of the five species. Correct classification % ranged between 79 and 94 % (Table 4).

### *Species richness*

The richest communities live in ponds without fish presence, with high sun exposure and near to species rich wetlands ( $F_{4,79} = 1.649$ ,  $P < 0.0001$ ,  $r^2 = 0.371$ , see Table 5). Environmental features alone explain only 17.5% of variance. Fish presence is an important limiting factor for newts and Italian tree frogs (Table 6); we found the green toad only in fish-free ponds too, despite it breeds in few wetlands to perform statistical analysis. The effect of fish presence on pool frog is marginally non significant. The pool frog and the Italian tree frog live in ponds with high sun exposure (Table 6). Only the presence of the Italian agile frog seems to be independent of sun exposure and fish presence. Fish presence is strongly associated with permanent water wetlands (G test:  $\chi^2 = 9.830$ ,  $df = 1$ ,  $P = 0.0017$ ): consequently, temporary water wetlands host the richest amphibian communities ( $U_{26,58} = 490$ ,  $P = 0.0065$ ).

Block	Variable	$\beta$	se	$P$
1	SUN EXPOS	+0.013	0.003	<0.0001
	FISH PRES	-0.445	0.209	<0.05
2	SP 1500M	+0.153	0.053	<0.01
	SP 250M	+0.589	0.283	<0.05
	constant	-0.292		

$\beta$ : multiple regression coefficient; se: standard error.

**Tab. 5.** Multiple regression model: effect of wetland features (block 1) and isolation (block 2) on species richness.

### Nestedness

Community system is ordered and shows a significant nested pattern at both spatial scale analysed using *null-model 0* and using *null model 1* (Table 7, Fig. 2). Neither *null-model 0*, nor *null-model 1* generated matrices with  $T^\circ$  values lower than system  $T^\circ$ . The rank of large-scale patches in the maximally packed matrix (Fig. 2B) is positively related to the number of wetlands in each patch ( $r_s = 0.621$ ,  $N = 11$ ,  $P < 0.05$ ). The species richness is positively related to the number of wetlands in a patch ( $r = 0.749$ ,  $N = 11$ ,  $P < 0.01$ ).

	SUN EXPOS		FISH PRES
	t (df=82)	$P$	Fisher exact test $P$
<i>T. carnifex</i>	0.941	>0.35	<b>0.045<sup>a</sup></b>
<i>T. vulgaris</i>	0.703	>0.35	<b>0.028<sup>a</sup></b>
<i>H. intermedia</i>	-6.098	<b>&lt;0.0001<sup>a</sup></b>	<b>0.0097<sup>a</sup></b>
<i>R. latastei</i>	0.656	>0.35	0.125
<i>R. s. esculenta</i>	-4.346	<b>&lt;0.0001<sup>a</sup></b>	0.085

a: significant association between species presence and high sun exposure/fish absence

**Tab. 6.** Association of each species with high sun exposure and fish-free wetlands.

Spatial scale	System $T^\circ$	<i>Null-model 0</i>		<i>Null-model 1</i>	
		$T^\circ$ mean (sd)	$P$	$T^\circ$ mean (sd)	$P$
Small	6.36°	45.34° (6.26)	<b>&lt;0.0001</b>	11.08° (1.45)	<b>0.0011</b>
Large	3.10°	36.83° (10.19)	<b>0.0003</b>	19.34° (7.39)	<b>0.028</b>

**Table 7.** Community nestedness measured at small and large spatial scale.

## Discussion

The complex distributional pattern of Amphibians in Lombardy floodplain is strongly related to wetland features and isolation. Both features seem to have similar importance for each species and for community richness, and they likely interact in a complex mode. Nestedness analysis shows the presence of a significant gradient of environmental stress between fragments (see Worthen *et al.*, 1998) at each spatial scale: this gradient reflects differences between fragments in environmental quality and isolation (Hecnar and M'Closkey, 1997a). Moreover, different species can have different response to habitat fragmentation since they have different movement capability and landscape connectivity can be different for different species. Our models do not show any relationship between wetland area and species richness. This is not surprising, since the biogeographic principle that larger areas support more species seems to have limitations in its application to wetlands. Several studies outlined the scarce importance of wetland surface for the richness of animal communities in wetlands (e.g. Pavignano *et al.*, 1990; Hecnar and M'Closkey 1998; Oertli *et al.* 2002; but see Lassen, 1975; Laan and Verboom, 1990), likely because other wetland features have a more direct effect on the species presence.

### *Habitat selection*

Wetland features are very important as determinants of amphibian distribution, because each amphibian species living in this area needs water for breeding. Our results about habitat selection of analysed species are consistent with previous studies (e.g. Pavignano *et al.*, 1990; Ildos and Ancona, 1994). Moreover, *Triturus vulgaris* avoids fish occupied wetlands (Table 4; Table 6): fish are some of the most important predators of newt larvae. *Triturus carnifex* prefers deep water: it is a nektonic species, and it needs well structured wetlands, with a well developed and structured vegetation (Pavignano *et al.*, 1990). Fish absence in temporary wetlands can explain the preference of *T. carnifex* for these water bodies (Table 4). *Hyla intermedia* too avoids wetlands with fish, and it prefers sunny wetlands. *Rana synklepton esculenta* is a very adaptable species, and may colonize very heterogeneous habitats (Pavignano *et al.*, 1990): the only significant preference is for high sun exposure (Table 4; Table 6). *Rana latastei* shows very different ecological requirements from other analysed species. It breeds during late winter, in river washes with deep water surrounded by broadleaved lowland forest, and it fixes spawn to submerged deadwoods. Since the river washes can be connected with the main river stream during flooding, they are frequently inhabited by fish.

### *Effects of sun exposure and fish presence on community structure*

The richest communities live in sunny, fish-free wetlands. Fish presence is a major threat for amphibians and it can drive amphibian community structure (Hecnar and M'Closkey, 1997b). Many fish predate amphibian larvae: newts, with nektonic active predator larvae, and tree frogs are some of the most endangered by fish presence (Bronmark and Edenham, 1994; Atkins, 1998). Species that can breed in fish inhabited wetlands have toxic/unpalatable tadpoles (i.e. toads: Semlitsch and Gavasso, 1992), or tadpoles acting anti predatory behaviour (i.e. pool frogs:

Semlitsch and Reyer, 1992). Fish presence in many wetlands is an anthropogenic factor of amphibian decline: humans frequently introduce fish for sportive fishing and ornamental/exotic species in naturally fish-free ponds. In Lombardy lowland this problem seems to be so intense that the observed effects of hydroperiod on amphibian communities are the reverse than in more natural landscapes: usually, permanent wetlands support the most diverse communities, since amphibians with longer larval periods are restricted to relatively permanent waters (Skelly *et al.*, 1999). Conversely, in the study area, temporary water wetlands host the richest amphibian communities, since no fish inhabit these wetlands. Fish abundance in permanent wetlands could be the cause of low number of species rich communities found (Fig. 2A) and of newts rarity: newt larvae require long time for development, but they do not live in fish occupied wetlands. Our results are consistent with those obtained in Britain (Beebee, 1997; Atkins, 1998): the largest, permanent ponds, that might host newt populations, are inhabited by fish, and newts, confined to temporary ponds, have declining populations.

Sun exposure is the second environmental features conditioning species abundance: shaded wetlands have lower light, lower dissolved oxygen, lower temperature and lower food resources (Werner and Glenmeyer, 1999). Low temperature and food scarcity mean lower tadpoles growth rate and survivorship (Bachmann, 1969; Skelly *et al.*, 1999; Werner and Glenmeyer, 1999), and therefore low tadpoles fitness.

The Italian agile frog is the only analysed species that does not show any preference for fish-free or sunny wetlands and, on small-scale nestedness analysis, *R. latastei* is the only idiosyncratic species (see Atmar and Patterson, 1993). Two independent analysis showed strong differences on ecological requirement between *R. latastei* and other amphibian species. Since Italian agile frog is endemic of Northern Italy and it is an endangered species, recently it became object of a number of conservation projects (Scali *et al.*, 2001). The results of this study show that conservation projects having *R. latastei* as target are of little help for many other species and conserving the richest communities can be unhelpful for conservation of *R. latastei*. Limitations associated with reliance upon species richness for conservation objectives are well recognised: when sub groups within a community exhibit opposite ecological requirements, the utility of species richness as a tool for reserve selection and habitat management is partially lost (Patterson *et al.*, 1996; Pearman, 1997; Hazell *et al.*, 2001). The limitation of species richness data for conservation is particularly strong when “idiosyncratic” species are the most endangered or endemic ones, like in this study.

### *Isolation*

In this landscape the least isolated wetlands are the richest in species, and the distance from the nearest occupied wetland or number of occupied wetlands in the same patch are good predictors for the presence of either newts and frogs: many species distribution appears “clustered” in metapopulation systems with different dimensions. One cluster could be used by one “patchy population”, with individuals regularly exchanging between close ponds, or could represent the habitat network of a metapopulation. The lack of spatial autocorrelation among the variables affecting amphibians distributions greatly strengthen the robustness of the observed relationship between wetland isolation and amphibians distribution: wetlands that are clustered do not have

similar species composition because favourable habitat is clustered. This pattern can be interpreted as the effect of isolation on population persistence probability (Sjorgen 1991): metapopulation theory predicts that there are thresholds conditions of patch density for metapopulation persistence on the landscape, below which a metapopulation and related subpopulations may become extinct (Hanski and Ovaskainen, 2000; Wilson *et al.*, 2002). Only the presence of the Italian tree frog does not seem to depend from distance to conspecific populations, but from community richness in the patch. Tree frogs can move across long distance using tree canopy (review in Stumpel and Tester, 1993), and it is possible that on the analysed spatial scale the effect of isolation on this specie is not evident; whereas, this species is more frequent in species-rich patches and it is possible that these patches have terrestrial habitats with better quality, because the quality of terrestrial habitat is very important for this strictly terrestrial species (Pavignano *et al.*, 1990; Ildos and Ancona, 1994). Further studies are required to better evaluate the role of terrestrial habitat on structuring the amphibian communities of this landscape.

#### *Landscape level persistence*

The community system is significantly nested at each considered spatial scale. The pattern is very strong, as low  $T^\circ$  values demonstrate. At small spatial scale, nestedness of communities is likely linked to nestedness of habitat suitability for species (see Davidar *et al.*, 2002, for a similar interpretation of nestedness). The wetlands that are richest in species host both the common and the rare species: conversely, the poorest communities host only the common species. The significant nestedness of the community system show that the rare species are more common than expected in the richest community, and more rare than expected in the poorest communities. In other words, if a wetland is suitable for the rare species it is suitable also for the common species, and not the other way round. Thus, the wetland with low isolation and high suitability for the amphibians are rich in species and host both rare and common species; conversely, a more isolated wetland and/or with habitat features less suitable (for example, a fish inhabited wetland) can host only the species less sensitive to isolation and habitat alteration: the commonest species in the landscape, the pool frog and the Italian tree frog, seem to met these requirements for several reasons.

First, the commonest species (pool frog) is very adaptable, it does not require large terrestrial habitat and it can live also in polluted water (Bucci *et al.*, 2000). Second, the pool frog and the Italian tree frog have a quick development, and they survive well in temporary wetlands. Third, these two species can easily cross the matrix of the landscape: pool frog can cross over long distances using water bodies (Tunner, 1992), and the very complex agricultural ditches web of Lombardy lowland allows this species to cross the landscape (Fig. 1). Moreover, since the pool frog is really abundant in this landscape, for this species the average distance between the occupied wetlands is lower: therefore, for the pool frog the landscape connectivity is intrinsically higher that for less abundant species. The Italian tree frog is an arboreal species, and it can move throughout the complex web of hedgerows existing in the Agricolo Sud Milano Park (Baietto *et al.*, 2002). The capability of Italian tree frogs to move across the matrix is confirmed by the data about its occurrence. The Italian tree frog breed in only 19 out of 84 wetlands (22.6%), however,

it is present in the 63.6% of large scale patches: this species breed in only few wetlands per large scale patch, but in almost all the patches. Therefore it seems that the Italian tree frog is able to move across the matrix to reach the suitable wetlands in each patch. Consequently, it is possible that for the pool frog and for the Italian tree frog a metapopulation system exists across the overall landscape: theoretical models and observational data demonstrate that long range dispersals have better persistence in a dynamic landscape, like cultural landscapes (Mader, 1990; Johst *et al.*, 2002). A similar overall pattern, where Italian tree frog and pool frog can cross barriers that are insurmountable for more terrestrial amphibians, were found in Northern Italy in the Lambro Natural Park by Ficetola and Scali (2002).

Conversely, newts and toads are the rarest species. They all are terrestrial during the post-breeding season: the effect of barriers on terrestrial amphibians is very strong, because they interrupt the metapopulation system (Hitchlings and Beebee, 1997, Rowe *et al.*, 2000), and hinder colonization of newly created ponds. It is remarkable that in our landscape isolation seem to be as important as environmental features for persistence of populations of these species, as high  $R^2_L$  values of the second blocks of the logistic regressions demonstrate. Moreover, newts can survive in very few wetlands, because of the scarcity of permanent wetlands without fish. The situation of *T. carnifex* seems to be very critical: the Italian crested newt development is slow, and larvae metamorphose on late summer (Griffiths, 1995), but in Lombardy lowland this species is confined to temporary, free-fish wetlands. In dry years *T. carnifex* reproduction could not be successful, and several consecutive reproductive failures can bring to extinction some newt populations (Atkins, 1998; Kupfer and Kneitz, 2000). Finally the common toad, the rarest species, requires large terrestrial, forested habitat patches (Romero and Real, 1996), and undergo to high mortality in landscapes with high road density (e.g. Cook, 1995): therefore, because of small forested fragments and high road density in the study area, *B. bufo* is confined to the largest one of studied terrestrial patch.

Similar conclusion are suggested by the nested pattern revealed by analysing the large scale patches. The patches richest in species are those with more wetlands (Fig. 2B): therefore, the wetlands in these patches are less isolated, and the presence of a local network of populations is possible. Moreover, a patch holding a high number of wetlands most likely hold also some suitable wetlands for amphibians. The consistence of results obtained analysing the community system at both small and large spatial scale strengthen the hypothesis that nestedness of communities reflects a gradient of environmental stress caused by human disturbance between patches: the wetlands can be placed along a gradient of decreasing quality, from the high quality, low isolation wetlands, hosting all the species, to the low quality, high isolation wetlands, hosting none or few species. Analogously, the large scale patches can be placed along a gradient from the patches holding many high quality wetlands to the patches holding few, low quality wetlands (Fig. 2B).

## Conclusion

The amphibian community assemblage on Lombardy lowland is related to two different forces, extinction and colonization, acting in a synergic mode on communities structure: degradation of wetland ecological features (like pollution and fish release) and landscape alteration (like

increased wetland isolation) cause the extinction of sensitive species from many patches. Moreover, only mobile and resilient species can cross the matrix, colonizing or re-colonizing the wetlands, preserving their metapopulation system and therefore allowing the long time species persistence in the landscape. If the process of patch degradation is not stopped, and if the landscape connectivity is not improved, the future will allow the persistence of very few species. Analogous extinction and colonization processes could be active worldwide in several other anthropogenic landscapes, driving the communities structure of vertebrates characterised by low mobility, like small mammals, reptiles and amphibians.

**Appendix.** Pearson product-moment correlations among environmental features and isolation features used for every species. N = 84 for all correlations. Abbreviations are in Tab. 1.

	AREA	WAT DEPTH	B SLOPE	WAT PERM	SUN EXPOS	SOIL	SUB WOOD	SUB VEG	FLOAT VEG	RIP VEG	GRASS	SCRUB	WOOD	FISH PRES	DUCKS PRES	DIST WET	N 250	N 1500	SP 250	
AREA	r	1																		
WAT DEPTH	r	0.108 0.326	1																	
B SLOPE	r	-0.202 0.066	-0.055 0.619	1																
WAT PERM	r	-0.073 0.507	0.388 <0.001	0.042 0.704	1															
SUN EXPOS	r	0.622 <0.001	-0.096 0.385	-0.156 0.156	-0.123 0.267	1														
SOIL	r	-0.058 0.599	0.124 0.263	-0.067 0.545	0.038 0.734	0.010 0.930	1													
SUB WOOD	r	-0.210 0.056	0.280 0.010	0.064 0.565	0.278 0.010	-0.447 <0.001	0.050 0.650	1												
SUB VEG	r	0.153 0.164	-0.276 0.011	-0.166 0.131	-0.371 <0.001	0.270 0.013	-0.078 0.482	-0.286 0.008	1											
FLOAT VEG	r	-0.057 0.609	-0.131 0.234	0.027 0.808	-0.012 0.917	0.156 0.156	0.214 0.050	0.143 0.194	0.026 0.814	1										
RIP VEG	r	0.026 0.816	-0.123 0.265	-0.095 0.389	-0.146 0.186	0.198 0.071	-0.109 0.323	-0.213 0.052	0.197 0.072	-0.239 0.028	1									
GRASS	r	0.110 0.320	-0.345 0.001	0.322 0.003	-0.270 0.013	0.362 <0.001	-0.243 0.026	-0.359 <0.001	0.278 0.011	0.078 0.480	0.290 0.007	1								
SCRUB	r	-0.079 0.477	0.361 0.002	-0.168 0.127	0.292 0.007	-0.338 0.002	0.352 0.592	-0.108 0.001	-0.127 0.329	0.104 0.250	-0.502 <0.001	0.436 0.005	1							
WOOD	r	-0.224 0.041	0.272 0.012	-0.076 0.489	0.138 0.209	-0.636 <0.001	-0.147 0.182	0.345 0.001	-0.264 0.015	-0.172 0.117	-0.087 0.433	-0.371 0.005	<0.001	0.436 0.225	1					
FISH PRES	r	-0.098 0.375	0.363 0.007	0.051 0.646	0.324 0.003	-0.116 0.294	-0.048 0.665	0.136 0.217	-0.057 0.608	0.080 0.471	-0.114 0.301	-0.216 0.048	0.259 0.017	0.040	1					
DUCKS PRES	r	0.084 0.446	0.184 0.094	-0.090 0.413	0.149 0.177	-0.055 0.622	-0.256 0.019	-0.200 0.068	-0.091 0.413	-0.137 0.213	-0.160 0.145	-0.091 0.409	0.161 0.145	0.137 0.212	-0.009 0.938	1				
DIST WET	r	0.109 0.324	0.169 0.124	-0.177 0.106	0.099 0.368	0.140 0.203	0.113 0.305	0.109 0.326	-0.067 0.545	-0.026 0.818	-0.067 0.542	-0.139 0.207	0.123 0.267	-0.100 0.367	0.066 0.552	-0.043 0.700	1			
N 250	r	-0.048 0.667	-0.237 0.030	0.264 0.015	0.016 0.884	0.071 0.520	0.342 0.001	-0.034 0.760	-0.209 0.057	0.284 0.009	-0.062 0.576	0.247 0.023	-0.270 0.013	-0.130 0.240	-0.225 0.040	-0.201 0.067	-0.184 0.093	1		
N 1500	r	-0.077 0.486	0.090 0.416	0.017 0.882	-0.031 0.782	0.021 0.851	0.467 <0.001	0.095 0.391	0.041 0.710	0.185 0.092	-0.038 0.729	-0.055 0.619	-0.029 0.794	-0.028 0.803	0.004 0.968	-0.155 0.160	0.249 0.022	0.287 0.008	1	
SP 250	r	-0.195 0.075	0.118 0.287	-0.027 0.808	-0.149 0.176	-0.244 0.025	0.031 0.782	0.186 0.091	0.031 0.777	0.056 0.610	0.023 0.836	-0.129 0.241	0.237 0.030	0.243 0.026	-0.080 0.467	-0.076 0.494	-0.138 0.209	0.014 0.897	0.323 0.003	1
SP 1500	r	-0.007 0.948	0.213 0.052	-0.195 0.076	0.003 0.975	-0.050 0.650	-0.096 0.385	0.239 0.029	0.018 0.873	-0.067 0.543	0.087 0.433	-0.202 0.066	0.279 0.010	0.114 0.303	-0.028 0.803	-0.022 0.843	0.325 0.003	-0.347 0.001	0.390 <0.001	0.491 <0.001



## 5. THE BIOLOGY OF THE ITALIAN AGILE FROG *RANA LATASTEI*

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### Systematic and intraspecific diversity

Class: Amphibia

Order: Anura

Suborder: Diplasiocelela

Family: Ranidae

Genus: *Rana* Linnaeus, 1758

Species: *Rana latastei* Boulenger, 1879

*Rana latastei* is a palearctic brown frog; *R. graeca* and *R. dalmatina* are the species more strongly related to *R. latastei*; *R. dalmatina* is sister group of *R. latastei* (Fig. 1). The oldest fossil attributed to *R. latastei* are from late Pliocene, and the divergence between *R. latastei* and *R. dalmatina* occurred likely in the early Pliocene (Veith *et al.*, 2003). This species is generally considered to have scarce or absent intraspecific variation (e.g., Lanza, 1983). However, recent studies regarding molecular genetic at nuclear markers showed that there is significant genetic variation among populations. Genetic diversity strongly decline from east to west (Fig. 2), suggesting that this species colonised the padano-venetian lowland from a Balcanic refuge after the last ice age (Angelone, 2002; Garner *et al.*, 2004a). Moreover, recent studies suggest that intraspecific variation could exist also for morphology and calls (Giacoma *et al.*, 2004; Marzona *et al.*, 2004).

### Morphology

#### *Adult*

A graceful brown frog with rather long legs (heel reach beyond snouth) and well separated dorsolateral folds. Throat consistently dark, usually with a narrow light central stripe that can seem a reversed T, and frequently a pink flush on throat and limbs. The light strip on hind upper lip stops abruptly under eye (Fig. 3). Snout often fairly pointed, but may be quite blunt. Eardrum not very large, well separated from eye and sometimes not clearly defined (Bruno, 1977; Lanza, 1983; Arnold and Ovenden, 2002). Dorsal colouring rather variable: upperparts greyish or reddish brown, often with some darkish blotches, especially a bar between eyes and a  $\Lambda$  shaped mark between shoulders (Lanza, 1983; Arnold and Ovenden, 2002). The intensity of pigmentation can change with the light, moisture and background environment: the darkest colours are most frequently observed in the daylight, while in the night the colours are more light. Frequently, the females are more reddish than do the males; moreover, females can have red or orange spots on the ventral side (Pozzi, 1980). No lichenous pattern on back or heavy blotches or flanks. Belly white, often marbled with grey at least anteriorly. Snout-to-vent length



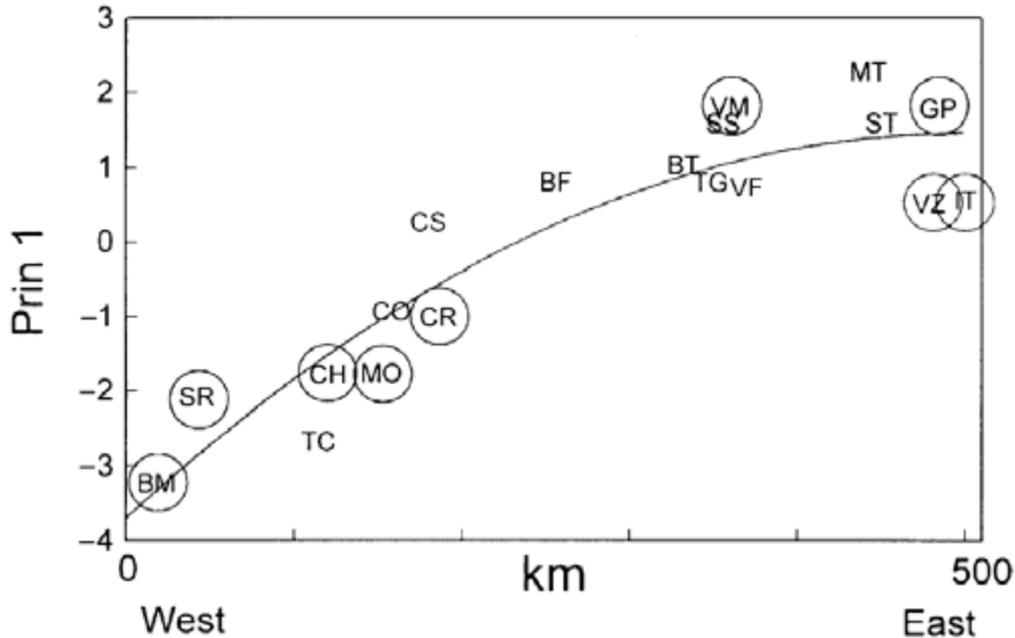


Fig. 2. Relationship between location along an east-to-west gradient and genetic diversity (Prin1) for *Rana latastei*. Genetic variability is the first principle component of the three measures of genetic variation described in text. Peripheral populations are encircled. From Garner *et al.* (2004a)

### *Sexual dimorphism*

Breeding males have dark brown nuptial pads on their thumbs, dark red-brown spots on the throat and orange to reddish colouring under the thighs (Lanza, 1983; Arnold and Ovenden, 2002). Usually, females are larger than males; however, males have larger and longer forelimbs (Lanza, 1983; Ficetola, 2001; Marzona *et al.*, 2004).

### *Eggs*

Females lay small clumps of spawn, with a diameter of ~ 10 cm, that are fixed to submerged woods at a depth of 10-40 cm. Clumps are more dense than those of *R. dalmatina*; classical studies report that each clump hold 95-900 eggs, more frequently 300-400 (Bruno, 1977; Lanza, 1983; Arnold and Ovenden, 2002). However, more recent measures reported 676-2720 eggs per clutch (average: 1278) (Bernini *et al.*, 2004b). It is possible that the earlier estimates are inaccurate, since the eggs are very close to each other and thus very difficult to accurately count.

### *Tadpole*

Pale brown with darker spots above, belly whitish, usually with transparent skin; tail crest usually whitish, with dark spots; the upper crest tapers abruptly to a very pointed tail tip. Spiracle on the left flank, rather close to the vent; vent on the left hand side of the tail; maximum length 50 mm; tail length is 2.5 times body length (Lanza, 1983; Nolert and Nollert, 1992; Arnold and Ovenden, 2002). Several authors report the oral disk to be a key features for the identification of



**Fig. 3.** Individuals of *R. latastei*. *a*: tadpole (Gosner's stage 24); *b*: metamorph; *c*: juvenile (Cassano d'Adda, MI); *d*: calling male (Monza); *e*: adult female (Zelo Buon Persico, LO); *f*: breeding pair (Monza).

*R. latastei* and *R. dalmatina* tadpoles: Lanza (1983) and Nollert and Nollert (1992) describe *R. latastei* tadpoles with the presence of three upper rows of labial teeth, while they describe *R. dalmatina* tadpoles with a middle tubercle in the upper mandible. However, more recent studies about animals from a large number of populations of the Po basin observed that ~ 63% of *R. latastei* tadpoles have a fourth row of labial teeth, while the middle tubercle is only rarely present in *R. dalmatina* tadpoles (Vercesi *et al.*, 2000; Barbieri *et al.*, 2000).

Further proposed features for the identification of *R. latastei* tadpoles from *R. dalmatina* are: the pigmentation of ventral skin (less pigmentation in *R. latastei*) and the distance between eyes (two times the distance between nostrils in *R. dalmatina*, 1.5 times or less in *R. latastei*) (Lanza, 1983; Nollert and Nollert, 1992).

No detailed studies exist about the behaviour of *R. latastei* tadpoles. Pozzi (1980) did not observe any evidence of gregarious behaviour, and hypothesized that tadpoles are more active by night.

### **Voice**

Four different voices has been described to be produced by *R. latastei* (Farronato *et al.*, 2000). The males produces a long drawn-out thin “mew”, rather like that of a cat, with long intervals between calls which occur every 20-60 seconds. This voice is produced under the water or at water surface, both by day and night (Nollert and Nolert, 1992; Farronato *et al.*, 2000; Arnold and Ovenden, 2002; Giacoma *et al.*, 2004). A different voice (“brum”) can be heard only under the water (Giacoma *et al.*, 2004), while the “release” call is a repeated “kek ... kek ... kek ... kek”.

### **Habits**

The overwintering last from November to late February – early March, but hibernation can be temporarily interrupted if the temperature rise up. It is assumed that the adults spent the winter underground, in burrows of small mammals (Pozzi, 1980; Lanza, 1983). *Rana latastei* breeds in wetlands (mainly ditches and ponds) within wooded areas, sometimes including slowly flowing parts of rivers. The males move to the wetlands in late February – early March, when the temperature is 6-8°C, and stay in the water some weeks (Pozzi, 1980; Dolce *et al.*, 1985, 1992; Scali, 1993; Vercesi *et al.*, 2000; Arnold and Ovenden, 2002). The females reach the breeding sites a few days after the males, staying in the wetlands only a few days or a few hours (Vercesi *et al.*, 2000). Usually, the females fix their egg masses to submerged woods: each female laid a single egg mass holding several hundreds of eggs; densities up to 150 egg masses per submerged wood has been recorded, suggesting than hundreds of different females can lay in the same small area (Pozzi, 1980; Dolce *et al.*, 1992). In nature, the eggs hatch after 14-25 days; the froglets metamorph from May to July (Pozzi, 1980). *Rana latastei* is a short lived frog: frogs reach the sexual maturity (body length  $\geq$  35 mm) when only one year old, and most frogs live less than 2-3 years; the maximum observed lifespan was 4 years (Guarino *et al.*, 2003; Guarino and Mazzotti, 2004).

The adults are active in the upland habitat (see below) from April to October, and they are more active in late summer – early autumn, during rainy days (Pozzi, 1980; Dolce *et al.*, 1985). *Rana latastei* is capable of long and rapid leaps (Arnolnd and Ovnden, 2002), but the adults likely spent most of their terrestrial activity underground, within the burrows of small mammals. The adults are only rarely observed more than a few hundreds of meters far from the wetlands (Pozzi, 1980; Dolce *et al.*, 1985).

## Feeding

*Rana latastei* feeds mainly on arachnida, esapoda (e.g., coleoptera, hymenoptera, orthoptera and lepidoptera), and sometime on terrestrial crustacea (isopoda) and gastropoda during the terrestrial activity (Pozzi, 1980; Dolce *et al.*, 1985); living underground, it feeds also on oligochaeta. In spring, during the breeding activity, it feeds also on molluscs and aquatic insects (Pozzi, 1980).

## Distribution and habitat

*Rana latastei* is endemic of the Padano-Venetian lowland and of adjacent areas. It live in Northern Italy, southern Switzerland (Canton Ticino: Mendrisiotto), southern Slovenia and in the westernmost corner of Croatia (Fig. 3) (SHI, 1996; Gasc *et al.*, 1997). It is a lowland species: it has been observed up the altitude of 700 m, but most populations live at altitudes lesser than 300 m (Lanza, 1983; Grossenbacher, 1997). In Lombardy, the highest altitude for this species is 520 m, but 95% of populations live at altitudes lesser than 400 m (Fig. 4) (Bernini *et al.*, 2004b).

*Rana latastei* is strongly associated to broadleaved riparian lowland woodlands of *Alnus glutinosa*, *Salix spp.*, *Pouulus spp.* and to the adjacent forests dominated by *Carpinus betulus*, *Quercus robur* and *Fraxinus excelsior*, with dense and moist understory (Pozzi, 1980; Lanza, 1983; Dolce *et al.*, 1985, Scali, 1993, Fasola *et al.*, 2000). Sometimes, it can also be found in popular plantations and in more open areas, such as grasslands and karstic areas (Scali, 1993, 1995; Bressi, 2001; Arnold and Ovenden, 2002).

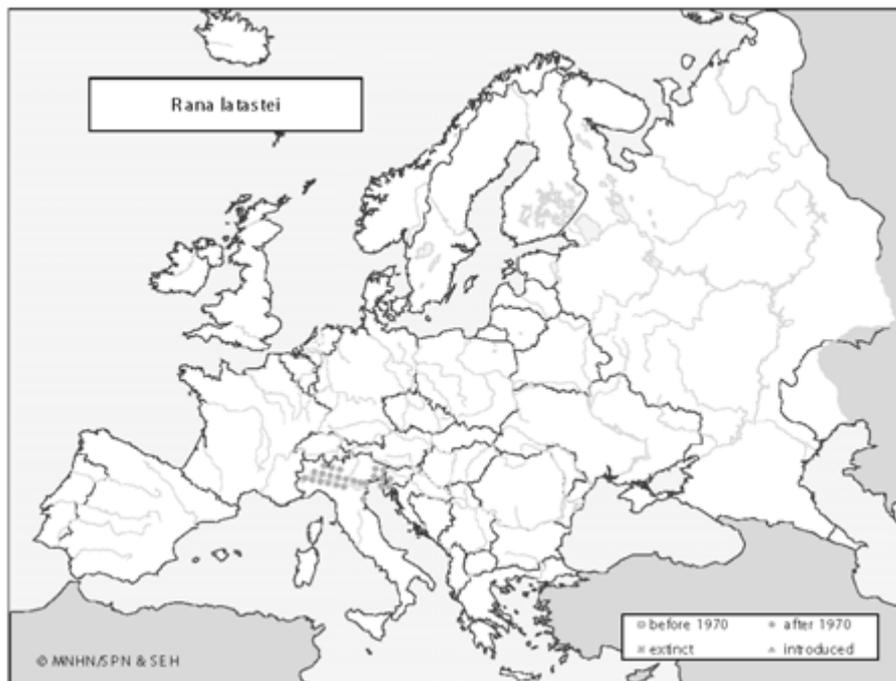


Fig. 3. Distribution of *R. latastei* in Europe. From Gasc *et al.*, 1997.

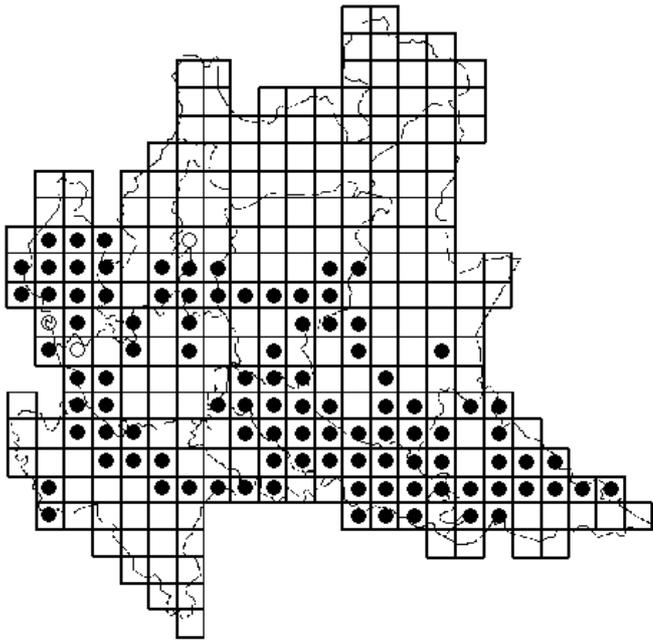


Fig. 4. Distribution of *R. latastei* in Lombardy. Empty dots: records not confirmed after 1985. From Bernini *et al.* (2004a).

### Conservation

*Rana latastei* has a small range; moreover, it is associated to lowland forests, an habitat threatened by human exploitation and fragmentation, thus it is a species of conservation concern (Andreone, 1992; Sindaco, 2000; Andreone and Luiselli, 2000; Gentilli and Scali, 2000). In the late 1980's and early 1990's only a few data existed about their distribution, and a very small number of localities with presence of *R. latastei* were known. Thus, *R. latastei* were considered to be globally threatened. In the European herpetological atlas (Grossenbacher, 1997) ~ 100 populations were recorded; actually, the number of known populations is certainly higher, since the program of monitoring promoted by the *Societas Herpetologica Italica* (SHI, 1996; SHI, in press; see also Bernini *et al.*, 2004a), and the number of records of *R. latastei* is still increasing (e.g., Sceglie, 2004). Thus, actually *R. latastei* is considered *near threatened* (NT) by the IUCN. However, the habitat of *R. latastei* is strongly threatened by human activities, and most populations are small and isolated. *Rana latastei* is protected by the Berna Convention; it is also included in the Habitat Directive of the European Union (EC 43/1992, annexes II, IV) as a species requiring rigorous protection and the designation of special protection areas, therefore plans are ongoing for their conservation (e.g., Gentilli *et al.*, 2003).

Recent studies observed that the western populations have low genetic diversity (Garner *et al.*, 2004a), and thus have reduced immunocompetence and may have reduced potential to evolve resistance to diseases (Pearman, 2003). This suggests a reduced probability of long-term survival of western populations, since they are more susceptible to introduced diseases, and because the fungus *Batrachochytrium dendrobatidis* has been observed in some *R. latastei* tadpoles (Pearman, 2003; Garner *et al.*, 2004b). Thus, the status of *R. latastei* can become very critical, and immediate actions are required to reduce the spread of this fungus across the River Po Basin.



## 6. INTERSPECIFIC SOCIAL INTERACTION AND BREEDING SUCCESS OF THE FROG *RANA LATASTEI*: A FIELD STUDY.

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*Ethology*, in press.

### Abstract

Interspecific reproductive interference can affect fitness related breeding performances, thus influencing fitness and distribution of populations. Laboratory studies have demonstrated social interference of *Rana dalmatina* males on *R. latastei* breeding females: the presence of heterospecific males reduced the percentage of viable embryos in *R. latastei* eggs. Here, we test if the negative effects of *R. dalmatina* males on *R. latastei* reproductive success occurs in nature. We compared the viable embryo percentage of eggs laid in nature from populations where *R. latastei* breeds alone with the viable embryo percentage of populations where *R. latastei* cohabits with *R. dalmatina*. We did not find significant differences in viable embryo percentage between *R. latastei* populations syntopic and allotopic with *R. dalmatina*, nor a relationship between relative abundance of heterospecifics and reproductive success. In nature the presence of heterospecific males does not seem to interfere with the reproductive success of *R. latastei*, contrasting with the results of experimental studies. Reproductive interference does not seem to be a major force influencing the distribution of these species. We suggest that the results of experiments dealing with complex interactions between animals should be validated on natural populations, to avoid the risk of interaction between experimental procedure and behaviour.

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### Introduction

The interspecific interaction during the breeding season can have important consequences on fitness, distribution and evolution of wild populations. For example, hybridisation can occur between sympatric, closely related taxa. In wild populations, hybridisation can occur between taxa that have not complete pre- or post-zygotic isolation: the hybrids can be sterile, or can be relatively less fit than their progenitors (Barton and Hewitt, 1985). When hybrids are less fit than their parents, selection favours the evolution of pre-zygotic isolation mechanisms (e.g., mating behaviours) that reduce heterospecific matings and, consequently, enhance reproductive isolation between species (a process called reinforcement) (Howard, 1993, Turelli *et al.*, 2001, Pfennig and Simovich, 2002). However, in certain cases and/or in certain habitats it is possible that hybrids have better performance: thus, the genotypes produced by hybridisation can have an important role in establish new evolutionary lineages (reviewed in Arnold and Hodges, 1995).

In the frogs of genus *Rana*, usually males stay in the breeding wetlands during the overall breeding season, attending for females. Since females frequently stay in the wetland only a few days or hours, the operational sex ratio can be very skewed, and at a given time the number of

males in the wetland is much higher than the number of females (e.g., Vieites *et al.*, 2004). More than one male can clasp the same female, and males can be very prone to mate also with heterospecific females, while females are more selective trying to breed only with conspecifics (Roesli and Reyer, 2000, Vieites *et al.*, 2004). Thus, hybridisation commonly occurs between several taxa belonging to the genus *Rana*. For example, the hybrids resulting by heterospecific matings between the pool frogs species *Rana lessonae* and *R. esculenta* are a distinct form traditionally called “*R. esculenta*” (Berger, 1967, 1968). *Rana esculenta* is an hemiclinal hybrid that eliminate the genome of one parental species from germline prior to meiosis, and clonally transmit the genome of the other parental species (Shultz, 1969). Usually, hybrids can persist in mixed populations by backcrossing with the parental genome they exclude, regenerating the hybrid genotype in the next generation (see Vorburger, 2000). At least in some cases, the hybrid form can be more fit than parents: it has been observed that *R. esculenta* are more abundant than the parental in areas with polluted water, suggesting that the hybrids are more resistant to pollution (Bucci *et al.*, 2000).

A different interaction between species that not interbreed or not produce viable offspring occurs when the sinthopy between two species modify their social environment, thus causing interspecific social interference. Interspecific social interference can negatively affect breeding related performance features, like the fertility of eggs or females, the frequency of pairing and the time budget available for breeding activity (e.g., Verrel, 1994; Takafuji *et al.*, 1997; Schultz and Switzer, 2001). For example the presence of the anemonefish *Amphiprion clarkii* suppress the reproduction in the juveniles of the closely related *A. perideraion* when the two species cohabit (Hattori, 2000). The presence of females of the salamander *Desmognatus fuscus* can cause an effect of “confusion” on the males of *D. ochrocephaelus*, disrupting their olfactory recognition of conspecific females. In presence of heterospecific females, *D. fuscus* males have a reduced probability of mating with conspecifics (Verrel, 1994). Thus, social interference during the breeding season can have important consequences on wild populations: if social interference causes a reduced performance in one species, it could affect their distribution similarly to other interspecific interactions, such as competition.

Here, we test if, in natural populations, the viable embryo percentage of the Italian agile frog *Rana latastei* is affected by the presence of the agile frog *R. dalmatina*. *Rana latastei* and *R. dalmatina* are two closely related species of brown frogs, and in the Padano-Venetian lowland (Northern Italy) these species, being syntopic, share many breeding sites. The mating between *R. dalmatina* and *R. latastei* does not produce viable offspring (Hettyey and Pearman, 2003), thus the hybridisation between these two species is impossible. However, recently Hettyey and Pearman (2003) demonstrated, under laboratory conditions, the potential of reproductive interference between *R. latastei* and *R. dalmatina*. They maintained females of *R. latastei* in outdoor experimental enclosures with an increasing relative abundance of males of *R. dalmatina* and revealed an increasing percentage of unviable embryos of *R. latastei* from *R. latastei* females in cages with an increasing percentage of heterospecific (*R. dalmatina*) males. Therefore, they hypothesized a social interaction between these species, and a cost in term of reproductive success of *R. latastei* when these two species breed close to each other. The aim of our study was to evaluate if, in nature, social interaction between these two species commonly occurs: we investigated if *R. latastei* populations syntopic with *R. dalmatina* show a reduced percentage of

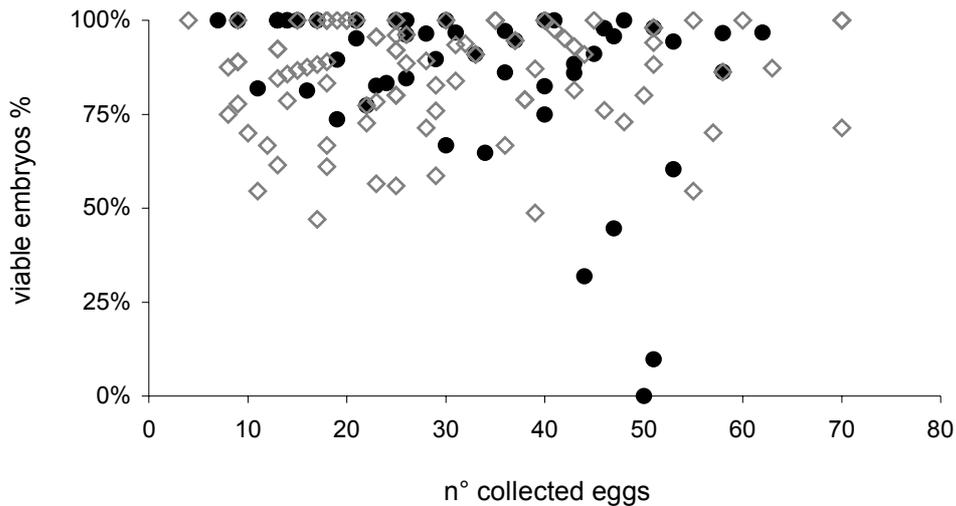
viable embryos. Despite these species have similar habitat requirement, in some area within the geographic range of both species only one of these frogs lives. It is possible that the interference between these species is acting at local scale in structuring their distribution (see Hofer et al, 2004 and references therein): this system is ideal to evaluate if social interference during the breeding season can be a force influencing the fitness and distribution of wild populations. The outcome of this study could also have important consequences for the management of *R. latastei* populations. *Rana latastei* is a threatened species, and actions of habitat management and relocation are founded by the European Union for their protection (Gentilli *et al.*, 2003). Therefore, if the reproductive interference is confirmed in nature, it should be kept into account during conservation plans.

## Methods

In Northern Italy, *R. latastei* and *R. dalmatina* breed from middle February to early April. Where these two species are syntopic, frequently *R. dalmatina* starts the breeding season a few days earlier than *R. latastei*. The males of both species stay in the wetlands for the overall breeding season. Conversely, the females stay in the wetlands only a few hours, leaving the water soon after the laying (Pozzi, 1980; Nollert and Nollert, 1992; Vercesi *et al.*, 2000). *Rana latastei* breeds in large, deep wetlands surrounded by woodland, with presence of submerged deadwoods to fix the egg masses. *Rana dalmatina* can breed also in more sunny, less deep wetlands (Vercesi *et al.*, 2000; Ficetola and De Bernardi, 2004). However, in several areas both species breed in the same wetland, and the overlap of breeding season is wide (Vercesi *et al.*, 2000).

We studied five populations of *R. latastei* in Lombardy (Northern Italy). All these populations breed in ditches within riparian woods of pedunculate oak (*Quercus robur*), hornbeam (*Carpinus betulus*), willow (*Salix* sp.) and black alder (*Alnus glutinosa*); the breeding sites are within the Adda Sud and Valle del Ticino Regional parks (altitude: 60 - 95 m). In two sites (N1 and N2, Adda Sud Park) only *R. latastei* live, in three sites (S1, S2, S3, Valle del Ticino Park) *R. latastei* is syntopic with *R. dalmatina*. In these three sites, both species breed contemporarily in the same waterbody, and egg masses of both species are frequently found very close to each other. The distance between population pairs within the same park was 10-30 km, a distance much greater than the know dispersal ability of *R. latastei*, therefore we assumed these populations to be independent. The distance between the two parks was approximately 40 km. Our populations live in wide ditches, and therefore it was not possible to quantify the relative abundance of males of these two species of frogs in the syntopic populations. However, we performed a semi-quantitative estimate of species abundance by counting the number of egg masses of *R. latastei* and *R. dalmatina*.

In March 2003, we gently removed a small portion (mean  $\pm$  SE:  $30.6 \pm 1.3$  eggs) from 26-32 *R. latastei* egg masses per site (Fig. 1, Fig. 2). Overall, we collected 4585 eggs from 150 different egg masses laid in nature, 94 from populations syntopic with *R. dalmatina* and 56 from allotopic populations. We transferred the eggs in 200 ml plastic containers and we returned them to the laboratory the same day, in order to reduce the risk that environmental differences between sites cause differences in the percentage of hatched eggs (Neckel-Oliveira, 2004).

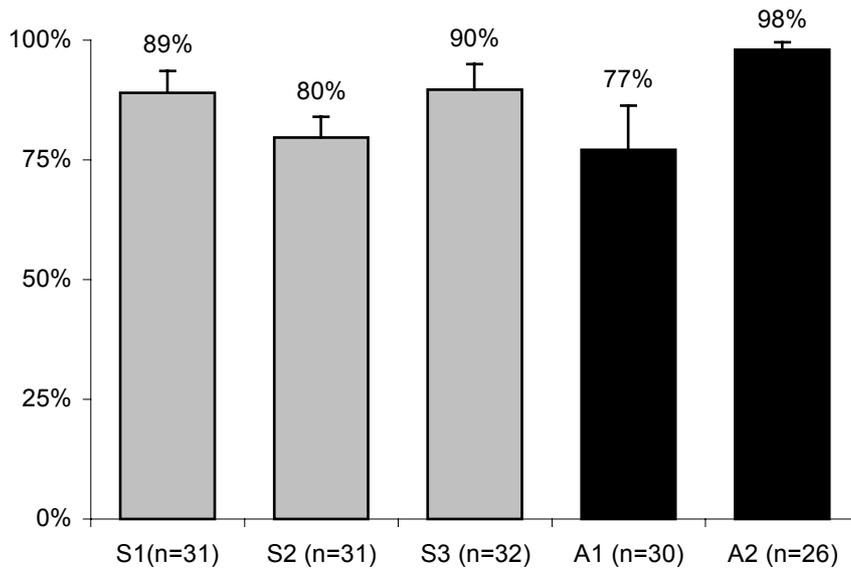


**Fig. 1.** Relationship between n of eggs collected per clutch and viable embryos %. Black dots: allotopic populations; empty diamonds: syntopic populations. Some points are superimposed.

To measure the percentage of viable embryos, egg masses were maintained in the same environment, with outdoor temperature and natural daylight photoperiod. We evaluated the percentage of viable embryos as number of tadpoles hatched from a spawn portion divided number of eggs in the portion. After they hatched, all the tadpoles were returned to their wetland of origin.

Because we manipulated the eggs when we removed the clutch portions, it is possible that our manipulation caused the death of some embryos and thus affected the percentage of hatched tadpoles. Such an effect could cause an increased egg mortality in the smallest egg portions, since in small portions an higher proportion of eggs was directly manipulated. We used logistic regression to test if the number of eggs removed from the clutches influenced the percentage of viable embryos.

Hetyey and Pearman (2003) observed a bimodal distribution of percentage of *R. latastei* egg viability when *R. dalmatina* males were present, with most of clutches showing either a very high or a very low fertility. We inspected the histograms of percentage of viable embryos, to evaluate the presence of this effect. We used nested analysis of variance to evaluate the effect of *R. dalmatina* presence and of the site of sampling on the percentage of viable embryos. The presence or absence of *R. dalmatina* was entered as a factor; site of sampling as a random factor nested within *R. dalmatina* presence. Since sample size was not homogeneous between sites, the degrees of freedom might be not integer. The percentage of viable embryos was arc-sin transformed to meet the assumption of analysis (Sokal and Rohlf, 1995). Tukey's post-hoc comparisons were used to evaluate pairwise differences between populations within group. After the analysis of variance, we performed a posteriori power analysis. Given the observed groups mean and the within group standard deviations, we estimated the observed power and effect size (Steidl *et al.*, 1997). The mean values are reported  $\pm$  SE; we performed statistical analysis using the programs SPSS 10.0.1 (© SPSS Inc 1989-1999) and R 1.9.1 (© 2004).

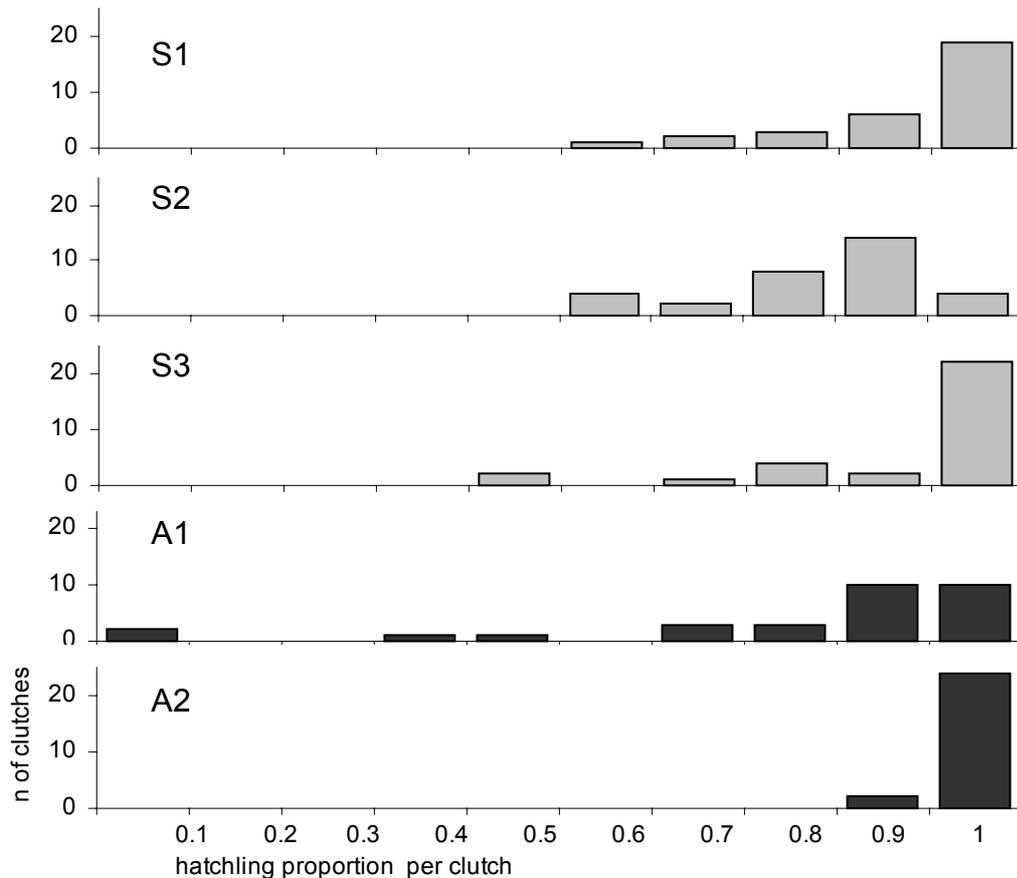


**Fig. 2.** Percentage of tadpoles of *Rana latastei* hatched from spawns from *R. latastei* populations syntopic (grey bars) and allotopic (black bars) with *R. dalmatina*. Between parentheses, number of egg masses analysed per population. Error bars equal 2 SE.

## Results

The percentage of viable embryos was not related to the number of eggs removed from the egg distribution of hatching percentage in syntopic populations (Fig. 3): in all populations, most of clutches showed hatching percentage  $> 80\%$ . Only 2 clutches showed hatching percentage under 10%, they were from the allotopic population A1; in the syntopic populations all clutches had hatching percentage  $> 40\%$ . In all populations, the frequency distribution of percentage of viable embryos was negatively skewed (population S1: skewness  $\pm$  SE =  $-1.398 \pm 0.421$ ; S2: skewness =  $-0.548 \pm 0.414$ ; S3: skewness =  $-1.690 \pm 0.414$ ; A1: skewness =  $-1.886 \pm 0.427$ , A2: skewness =  $-2.624 \pm 0.456$ ; see Fig. 1). Conversely, the pattern of kurtosis was not consistent among populations (population S1: kurtosis  $\pm$  SE =  $1.092 \pm 0.821$ ; S2: kurtosis =  $-0.192 \pm 0.809$ ; S3: kurtosis =  $2.324 \pm 0.821$ ; A1: kurtosis =  $3.234 \pm 0.833$ , A2: kurtosis =  $6.627 \pm 0.887$ ).

The average percentage of viable embryos per egg mass  $\pm$  SE was  $86.8\% \pm 2.8$  (N = 56, range: 0-100%) in allotopic populations and  $86.0\% \pm 1.4$  (N = 94, range: 47-100%) in syntopic populations. Average percentage of viable embryos per population ranged between 77.1% (population A1) and 98.0% (pop. A2) in allotopic populations and between 79.7% (pop. S2) and 89.7% (pop. S3) in syntopic populations (Fig. 2). We observed significant variability within the groups of syntopic and allotopic populations ( $F_{3,145} = 18.410$ ,  $p < 0.001$ ). Within the allotopic group, the population A1 showed significantly lower percentage of viable embryos than the population A2 (Tukey's post-hoc:  $p < 0.001$ ). Within the syntopic group, populations S1 and S3 showed higher percentage of viable embryos than population S2 ( $p < 0.02$  in both comparisons) but not significant differences were observed between populations S1 and S3 ( $p = 0.931$ ). Differences among syntopic populations in the percentage of viable embryos do not seem to be related to the relative abundance of *R. dalmatina*. When we performed sampling, in the



**Fig. 3.** Frequency of hatching percentage in spawns from *R. latastei* populations syntopic (gray bar: S1, S2, S3) and allotopic (black bars: A1, A2) with *R. dalmatina*.

population S1 we counted about 31 *R. latastei* clutches and about 35 *R. dalmatina* clutches; in the population S2 we counted about 90 *R. latastei* clutches and about 200 *R. dalmatina* clutches; in the population S3 we counted about 32 *R. latastei* clutches and about 150 *R. dalmatina* clutches. Thus, the syntopic population with the lowest percentage of viable embryos was not the population with the higher apparent relative abundance of *R. dalmatina*, while the populations S1 and S3 shared similar percentage of viable embryos despite strong differences in the apparent abundance of *R. dalmatina*.

We did not observe significant differences in percentage of viable embryos between syntopic and allotopic populations ( $F_{1,3.011} = 0.129$ ,  $p = 0.743$ ). For this latter analysis, a-posteriori power and estimated effect size were very low (power = 0.057; effect size = 0.174); the between groups variance (0.00187) was much lower than the within groups variance (0.123). The difference between groups were very small: the weighted averages for the two groups (86.8% for allotopic and 86.4% for syntopic) differ by only 0.4%.

## Discussion

Our analysis did not reveal any difference in percentage of viable embryos between populations of *R. latastei* syntopic and allotopic with *R. dalmatina*, and the percentage of viable embryos does was not related to the relative abundance of the two species. For example, the population S3 live in an environment with an higher relative abundance of heterospecifics than both the populations A1 and S2, but egg masses of this population have an higher percentage of viable embryos. A possible limitation of our study is the low number of investigated populations, since a low number of populations reduces the power of analysis. However, a posteriori power analysis showed a very low effect size (0.174), and the weighted averages of two groups differ by only 0.4%: it seems to be unlikely that the presence of heterospecific males have a biological effect on the percentage of viable embryos (Steidl *et al.*, 1997). Hettyey and Pearman (2003) observed a reduction of reproductive success also in experimental containers with a ratio *R. latastei* /*R. dalmatina* males abundance = 5:1, and a percentage of viable embryos under 20% when the ratio was 1:5. Conversely, despite in our syntopic populations the ratio (*R. latastei*) / (*R. dalmatina*) abundance was always  $\leq 1$ , in none of these populations we observed an average viable embryo percentage  $< 75\%$  or a bimodal distribution of hatching percentage (Fig. 2, 3). Therefore, it seems unlikely that social interference between *R. latastei* and *R. dalmatina* commonly occurs in wild populations and has an effect comparable to those observed under laboratory conditions, thus influencing distribution of these species.

It should also be noted that the average percentage of viable embryos we observed ( $\geq 77\%$  in all populations) is comparable to those measured using similar methods in other wild populations of the genus *Rana*. In a population of *R. temporaria* the average percentage of fertilized eggs was 72.9% in absence of any interaction by conspecific or heterospecific males (Vieites *et al.*, 2004). Thus, the unviable embryos we observed are likely the proportion of unfertilised eggs that currently occurs in frog populations, and they are not the outcome of heterospecific sperm competition.

An alternative explanation of our data could be that females from the syntopic populations laid eggs with inherently higher fertility and the negative effect of *R. dalmatina* presence compensates this advantage. However, we do not have any evidence of factors that could negatively affect the fertility of allotopic populations. All populations live in similar habitats (ponds have similar depth and surface; riparian woodlands have comparable composition), therefore there is not any obvious gradient of environmental quality to relate with the percentage of viable embryos. Alternatively, if a geographical trend of genetic diversity exists, and if allotopic populations experience lower genetic diversity, than the inbreeding depression could affect the allotopic populations, and the presence of *R. dalmatina* could affect the syntopic populations, resulting in similar reproductive success. Despite we do not have data on population genetic diversity, we can reject this hypothesis. *Rana latastei* shows a wide scale geographic trend, with genetic diversity decreasing from east toward west (Garner *et al.*, 2004a). The allotopic populations (Adda Park) are more eastern than do the syntopic ones (Ticino Park). Therefore, if the effects of interaction with *R. dalmatina* were additive with the effect of geographic genetic trend, any differences between the syntopic and the allotopic group would be evident.

The effects of social interaction between two frog species on reproductive success was not evident in the wild populations we studied, despite a laboratory study demonstrated its potential. There are various explanations for this discrepancy. First of all, in nature the *R. dalmatina* males have access to conspecific females and thus they could have a reduced propensity to try copulation with *R. latastei* females. Moreover, it is possible that the limited space available to the males in the experimental containers physically increased the interactions between species. Again, in nature many tenths of *R. latastei* females frequently lay their eggs clumped to the same submerged wood (Pozzi, 1980). It is possible that these aggregations of conspecifics reduce the opportunity of interaction for heterospecific males. Further studies are required to evaluate the niche segregation mechanisms between these two closely related species of frogs. Finally, our study populations were not the same studied by Hettyey and Pearman (2003): populations living in even slightly different sites may differ in their behaviour, for example because they are adapted to different environments (Freidenburg and Skelly, 2004; Ficetola and De Bernardi, 2005). Indeed, interspecific matings can be a cost for both sexes, since they do not produce viable offspring. Despite males can try mating with more than one female, the fitness cost of interspecific matings for males can be a reduced opportunity for mating with conspecifics females, thus in our populations selection may have act to avoid interspecific matings (Pfenning and Simovich, 2002).

If the presence of *R. dalmatina* does not seem to affect the amount of viable eggs, what could be the cause of the significant variability among populations? We did not analyse other environmental differences among populations, and we did not observe a regional trend of viable embryos percentage consistent with the trend of genetic diversity observed by Garner *et al.* (2004a). Despite a genetical analysis of each population was beyond the aims of this study, we speculate that, at local scale, differences in genetic diversity could explain the differences in viable eggs % among populations. The populations of *Rana latastei* of Lombardy are affected by low genetic variability: the genetic diversity measured in three populations of western Lombardy (including our population S2) is lower than the average genetic diversity of *R. latastei* (Garner *et al.*, 2004a). Moreover, most of existing populations of *R. latastei* live in the least isolated wetland, suggesting that some isolated populations have already gone extinct (Ficetola and De Bernardi, 2004). Therefore, it is possible that the differences in viable embryos percentage are caused by negative genetic effects in some populations, such as inbreeding depression (Keller and Waller, 2002).

The use of laboratory studies is based on the assumptions that laboratory experiments allow more accurate experimental manipulation and give realistic results (Huston, 1999; Skelly and Kiesecker, 2001). Of course, a large number of studies confirmed in the field the results obtained under experimental conditions (see Drenner and Mazumder 1999, and references therein; Mathis *et al.*, 2003). For example, the presence of the salamander *Plethodon jordani* negatively affected the distribution of *P. glutinosus* in experimental studies, and the field observation confirmed that the negative interspecific interaction causes at least partially the scarce overlap of their altitudinal distribution (Hairston 1949, 1980). However, in some cases field and laboratory experiments resulted in different outcomes. Skelly (2002) observed that the presence of tadpoles of the ranid frog *Rana sylvatica* negatively affects growth and development rate of the hylid frog *Pseudacris crucifer* in mesocosms but not in the field. Bezemer and Mills (2003) observed that

the parasitoid *Mastrus ridibundus* increases brood size with host size in the field, but not in laboratory. Verrel (1990) recorded higher frequency of heterospecific mating between two salamander species in small rather than in large experimental arenas. In our study, interference between *R. dalmatina* and *R. latastei* does not seem to have major influence on the percentage of viable embryos eggs of *R. latastei*, despite the potential demonstrated in laboratory. In general, a number of studies revealed poor concordance between laboratory and field experiments involving interaction between individuals or species. This poor concordance could be due by the stress caused by laboratory conditions, by different interaction frequencies among individuals, or by unpredictable effects of the experimental environment on the animals (Verrel, 1990; Skelly, 2002; Bezemer and Mills, 2003; Joron and Brakefield, 2003). Therefore, in some cases patterns of interspecific interaction derived from experiments could be different from those experienced by natural populations: there is the risk to make incorrect inferences and to attribute to interspecific interaction patterns that could be due to other factors (Skelly and Kiesecker, 2001). Experiments are an essential tool in all sciences. However, we stress the importance to validate in nature the outcome of experimental studies (Huston, 1999, Mathis *et al.*, 2003). Field validation can be very important for studies involving complex interactions, such as ecological, social and behavioural (Kohler and Wiley, 1997).



## 7. LOSS OF FITNESS IN ISOLATED POPULATIONS: LARVAL SURVIVAL IN THE FROG *RANA LATASTEI*, AND SUGGESTIONS FOR THE MANAGEMENT OF POPULATIONS

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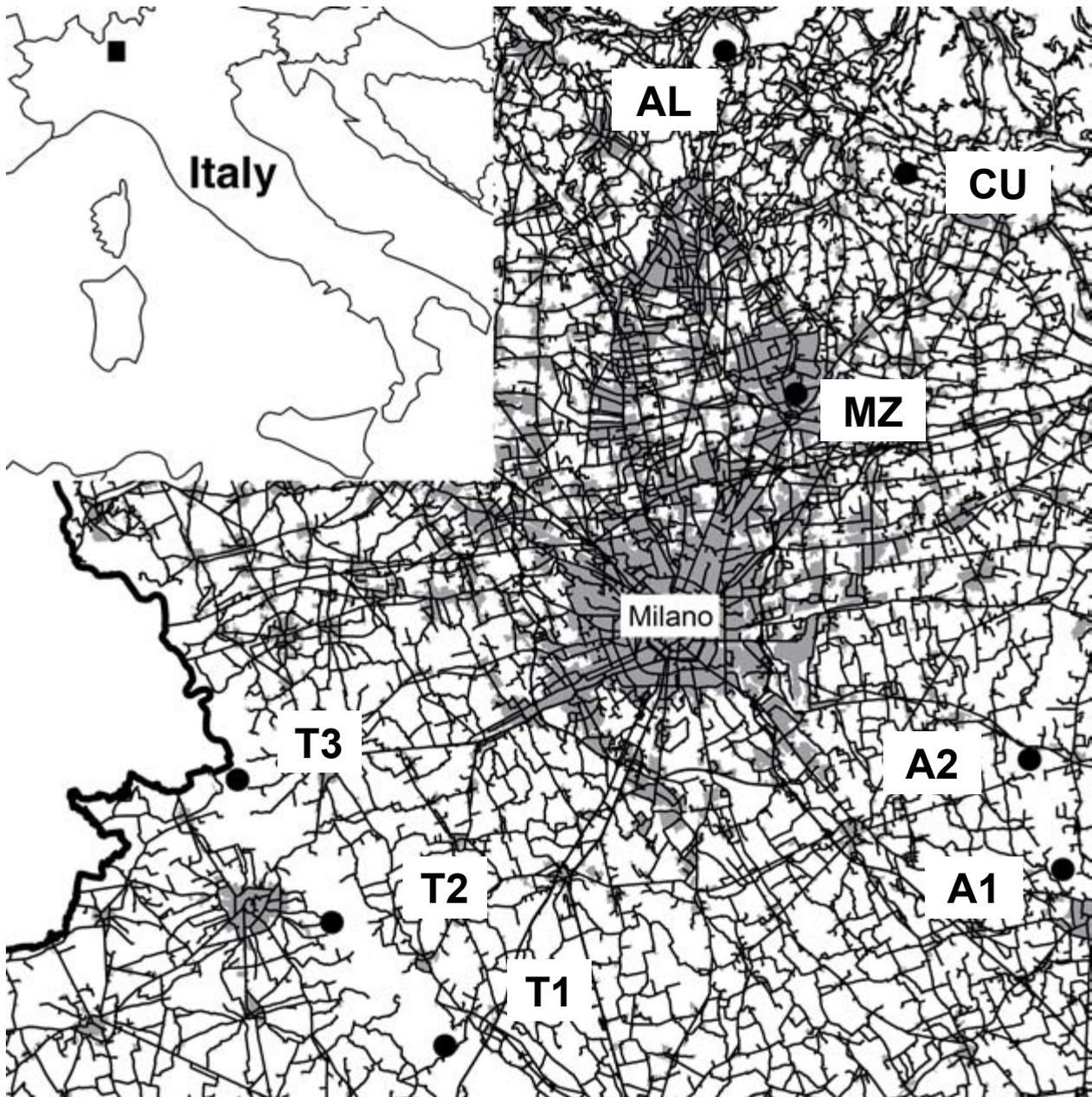
### Abstract

Isolated populations can lose genetic diversity: this phenomenon can result in loss of fitness of individuals. This loss of fitness (inbreeding depression) can be a major threat to biodiversity, since can cause the extinction of isolated populations. We investigated survival from hatch to metamorphosis in three isolated populations and in five non isolated populations of *R. latastei*, by rearing under standard environmental conditions eggs collected in nature. Egg masses from isolated populations have a reduced survival during pre-hatch development stage and during stages from hatch to free swimming tadpoles (Gosner's stages 18-25). We did not observe significant differences among populations in survival during the growth or during the metamorphic climax. In absence of predation or competition for food, only 20% of embryos from isolated populations survived to metamorphosis, suggesting that inbreeding depression in our populations is strong. Management actions are required to increase the viability of populations; the local adaptations demonstrated by contemporary studies suggest to carefully evaluate which conservation strategies might be more appropriate.

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### Introduction

Habitat fragmentation is a major cause of loss of biodiversity (Wilcox and Murphy, 1985). First, populations living in isolated habitat patches suffer an increased risk of extinction for environmental and demographic stochasticity. For example, an isolated patch can become temporarily unsuitable for a species because of an environmental fluctuation. If the patch is isolated, the recolonisation of the patch (or the rescue effect on the surviving individuals) is prevented, and the patch will be uninhabited by the extinct species also if - after the environmental alteration - the patch become newly suitable (Den Boer, 1990; Ficetola and De Bernardi, 2004). A further negative effect that can occur in isolated populations is the loss of fitness caused by the loss of genetic diversity (Hedrick, 2001; Keller and Waller, 2002). In small, isolated populations natural selection is less effective and deleterious mutations tend to accumulate (Lynch *et al.* 1995). Moreover, genetic drift causes the erosion of genetic diversity and the random fixation of unfit alleles (genetic load: Hedrick 2001). Finally, the restricted opportunities for mating increases the frequency of mating among relatives and thus the frequency of individuals that are homozygous for alleles identical by descent (inbreeding). Altogether, these genetic mechanisms threaten the long term survival of populations, and are called "inbreeding depression" (Keller and Waller 2002).



**Fig. 1.** Study area. Black dots: sampled populations; grey: urban areas; black lines: routes. The bold black line represent the boundary of Lombardy Region. Note the differences in route density between the areas were isolate (AL, CU, MZ) and not isolated (T1, T2, T3, A1, A2) populations live.

Since isolated populations suffer a reduced fitness, inbreeding depression should be kept into account in management plans. Species that have a reduced mobility and that usually live within metapopulations can be strongly susceptible to the negative effects of isolation (Hanski *et al.*, 1995). A synergism is possible between environmental and demographic stochasticities and the inbreeding depression: the risk of extinction of isolated populations could be exacerbated by the contemporary possibility of stochasticities and of inbreeding. Anthropogenic elements of landscape, such as routes or urban areas, can be insurmountable by small, terrestrial animals (such as ground beetles, small mammals, amphibians and reptiles). Molecular genetic studies have revealed the existence of genetic differences among populations separated by routes or

Population	Isolated	N of observed egg masses	N of samples	Distance from the nearest <i>R. latastei</i> population know	Hatch proportion	G25 proportion
T1	N	31	31	-	0.89 ± 0.02	0.89 ± 0.05
T2	N	250	32	-	0.80 ± 0.02	0.91 ± 0.04
T3	N	31	31	-	0.90 ± 0.03	0.89 ± 0.05
A1	N	26	26	-	0.98 ± 0.01	0.99 ± 0.01
A2	N	1000	30	-	0.77 ± 0.05	0.92 ± 0.05
MZ	Y	155	32	12 km (Lomagna)	0.48 ± 0.06	0.62 ± 0.08
AL	Y	41	30	3 km (Rogeno)	0.54 ± 0.05	0.36 ± 0.09
CU	Y	10	5	4 km (lago di Sartirana)	0.38 ± 0.12	0.60 ± 0.24

**Tab. 1.** Descriptive statistics of eight sampled populations. Means are reported ± 1 standard error.

small amounts of urban areas (Reh and Seitz, 1990; Hitchlings and Beebee, 1997; 1998; Prosser *et al.*, 1999; Gerlach, 2000; Rowe *et al.*, 2000; Vos *et al.*, 2001; Keller *et al.*, 2004): these animals are strongly susceptible to the effects of isolation. In human dominated landscapes, most of residual patches are isolated within an altered matrix, thus long term isolation could result in the extinction of a large number of populations, and consequently in a strong loss of biodiversity (Hanski and Gilpin, 1997; Huges *et al.*, 1997).

However, the effects of long term isolation on populations are not well documented. Indeed, despite several studies observed a reduced gene flow among populations, usually populations can not be considered completely isolated, since the flow of individuals across barriers or across the matrix is assumed to be possible (Haila, 2002). Here, we studied the effects of isolation populations of the frog *Rana latastei* that could be considered completely isolated. The distribution of this species in Lombardy (Northern Italy) is well know, since a ten years census program accurately monitored this region (Bernini *et al.*, 2004a). After the end of the breeding season *R. latastei* is a terrestrial species, however, it was never observed more than a few hundred meters further from the breeding wetland;. Three out of our study populations (MZ, AL and CU: Fig. 1) can be considered completely isolated from conspecific populations. The closest conspecific populations are respectively 12, 4 and 3 Km far (Tab. 1). Moreover, main routes separate the populations AL and CU from the closest conspecific populations. For the population MZ the isolation is, if possible, stronger. This population live in a urban park, surrounded since 1805 by a 3-m-talled wall, thus insurmountable by terrestrial amphibians (see Ficetola and Scali, 2002). Thus, for these three populations isolation is likely to be complete. We compared these isolated populations with five non isolated populations living in large riparian woodlands (see Methods, below). We measured, under standard laboratory conditions, survival of embryos and tadpoles until the metamorphosis, since survival can be considered an important component of fitness.

### Population sampling

We sampled eight populations of *R. latastei* in Lombardy, Northern Italy (Fig. 1), in march 2003, during the breeding season. We sampled all the egg masses we were able to find in a wetland,

until a maximum of 35-37 samples: a small portion ( $36.14 \pm 1.28$  eggs) of each clutch was gently removed and kept in plastic tanks. We have previously demonstrated (see Chapter 6) that this sampling do not influence survival of embryos. Egg samples were immediately returned to the laboratory. All the population were located in natural Parks; three of them were settled near to the Ticino River, in the Ticino Valley Natural Park (T1, T2, T3), two near to the Adda River (A1, A2), in the Adda Sud Natural Park. Both this river are relatively large, and they have an almost continuously wooded riparian area; wetlands near to the river are washes frequently inundated during flood. These wetlands are part of complex wetland systems, in each one of them probably live a metapopulation of *Rana latastei* (Ficetola and De Bernardi, 2004; Bernini *et al.*, in press), thus we called these five populations “not isolated”. The remaining tree populations live in residuals of fragmented woods in the Lambro Valley Natural Park and in the Montevecchia Valle del Curone Natural Park. One of this population (MZ) is settled in a large urban park, fully surrounded by a wall 3 m tall since 1805 (Ronzoni, 1998), and it is unlikely that a terrestrial amphibians cross this barrier (Ficetola & Scali, 2002). The remaining two populations (CU, LA) live in wood patches fully surrounded by streets, without large river crossing the boundaries of the patch, and far from any know population (Tab. 1). The contemporary presence of insurmountable barriers and the distance from the nearest conspecific population allow us to assume these populations to be “isolated”

All the isolated populations breed in a single wetlands in the patch, and we therefore assumed that none of them is part of a metapopulation.

### *Fitness assessment*

We used the Gosner's (1960) tables to evaluate the developmental stages of larvae. To evaluate the fitness of *R. latastei* populations across developmental stages from egg laying to metamorphosis, we divided development into four intervals: interval 1, egg laying-hatch; interval 2, hatch-free-swimming tadpole (stage 25); interval 3: tadpole growth, from free swimming tadpole to the beginning of metamorphosis (skin over the forelimbs transparent, stage 41); interval 4: metamorphosis, from the beginning of metamorphosis at the tail resorption (stage 45). Therefore, in the text, we refer to this four intervals as hatch, G25, tadpole growth, metamorphosis.

### *Experiment 1. Survival until the stage 25.*

We collected a total of 7335 eggs from 218 different clutches. Since each females laid a single clutch each year, we assumed these clutches to be independent replicates. Immediately after collection, each clutch portion were kept in a plastic tank filled with 0.2 l of aged tap water. All the tanks were randomly sorted in the same room, with natural photoperiod and at outdoor temperature, and were monitored daily. For each clutch, we recorded HATCH % as the percentage of larvae that emerged from their egg, and G25 % as the percentage of hatched larvae that survived until free-swimming stage (stage 25). After the end of hatching event (two weeks after the last hatching in a clutch), we randomly collected a subsample of the not-hatched eggs. We examined eggs under a stereomicroscope, to evaluate if the egg started to develop. We

examined a total of 590 eggs coming from 25 clutches, 12 clutches from non isolated and 13 from isolated populations.

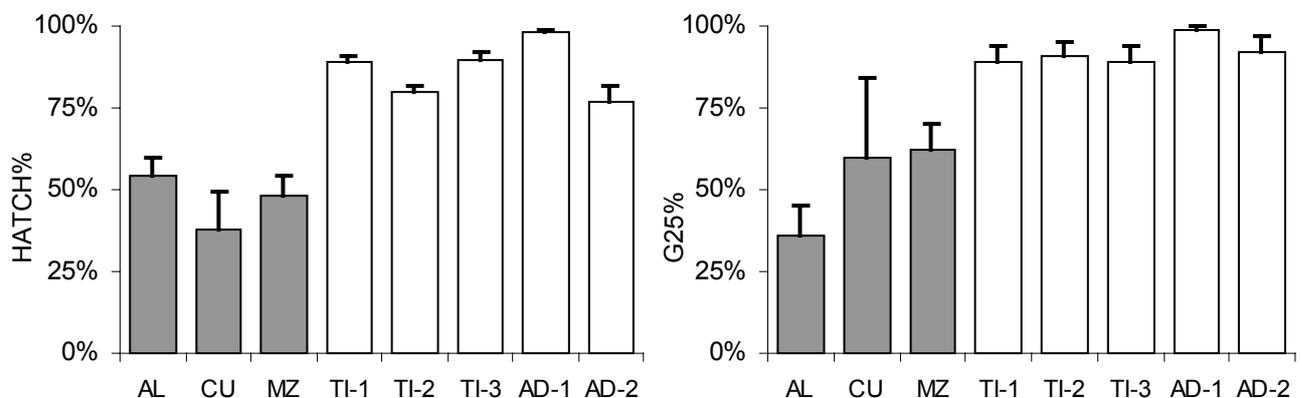
### *Experiment 2. survival from stage 25 to metamorphosis*

On reaching the free-swimming development stage, we placed in 23 plastic containers 230 tadpoles from 23 further clutches (Populations AL, MZ, A2, T2: 5 clutches from each population, 10 tadpoles from each clutch; population CU: 3 clutches, 10 tadpoles from each clutch). Each container was filled with 1.5 l aged tap water; containers were randomly sorted over the same bench of the laboratory (temperature: constant 20°C, 12 h natural daylight). All tadpoles from the same clutch were reared in the same container. Tadpoles were fed *ad libitum* with rabbit chow pellet and lettuce, water was changed weekly. We monitored containers daily to detect dead tadpoles; when tadpoles approached to metamorphosis (developmental stage 42) water level were reduced to avoid the risk of die by drowning; at developmental stage 43 metamorphs were kept in smaller plastic container holding 0.5 cm of water, allowing them to sort out of the water for aerobic respiration. On reaching developmental stage 45 (almost complete tail resorption) the experiment ended. At the end of the two experiments, we liberated all the surviving tadpoles or froglets in their natal wetland.

### *Data analysis*

We used mixed models to test the effects of isolation and population of origin (POP) on HATCH% and G25%. Isolation (y/n) was entered as a fixed factor; population of origin as a random factor nested within isolation. Models were performed on arcsine transformed data to better met the assumption of analysis (Sokal and Rohlf, 1996).

Data on survival during growth and metamorphosis failed to met the assumptions of parametric analysis, thus we analysed them using non-parametric tests.



**Fig. 2.** Hatch% and survival from hatch to stage 25 (G25%) in isolated (grey bars) and not isolated populations of *R. latastei* (white bars). Error bars equal standard errors.

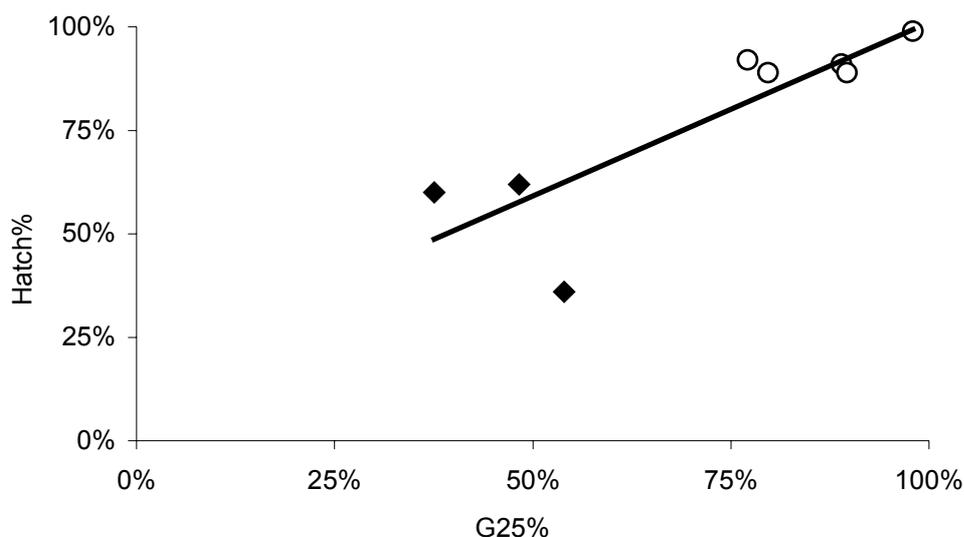
	<i>F</i>	<i>df</i>	<i>P</i>
HATCH%			
Isolation	19.618	1, 7.351	<b>0.003</b>
Population(Isolation)	7.284	6, 209	<b>&lt;0.0001</b>
G25%			
Isolation	26.395	1, 12.633	<b>0.0002</b>
Population(Isolation)	1.714	6, 205	0.119

**Tab 2.** Differences among populations in percentage of hatched eggs (HATCH%) and survival from hatch to development stage 25 (G25%): results of mixed model ANOVAs

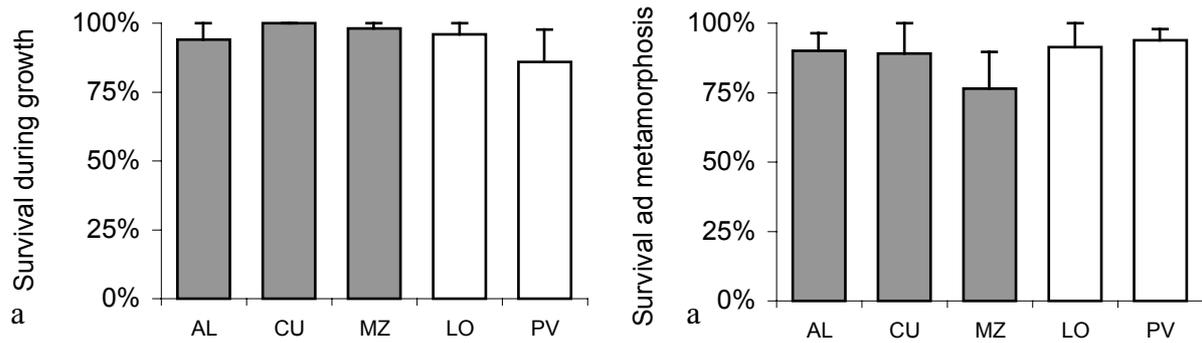
## Results

### *Experiment 1*

Isolated populations suffered a reduced HATCH% and G25%, if compared with non isolated populations (Tab. 1, Fig. 2). Average HATCH% was always above 75% in non fragmented populations, and always less than 55% in isolated populations; G25% was always above 88% in non isolated populations and always less than 63% in isolated populations. Differences between the isolated and non isolated groups in HATCH% and G25% were significant; we also observed significant variation among populations within group in HATCH%, while within group differences in G25% were not significant (Tab. 2). The populations with higher average HATCH% were those with higher G25% (Pearson's correlation:  $r = 0.842$ ,  $N = 8$ ,  $P = 0.0088$ ): the populations with higher HATCH% were those with higher G25% (Fig. 3). However, after keeping into account differences among populations, we did not observe any relationship



**Fig. 3.** Relationship between average HATCH% and average G25% in *R. latastei* populations. Black diamonds: isolated populations; empty circles: non isolated populations.



**Fig. 4.** Average survival during larval growth (a) and at metamorphosis (b) of tadpoles from five *R. latastei* populations. Grey bars: isolated populations; white bars: non isolated populations.

between HATCH% and G25% of the same clutch ( $F_{1,204} = 0.05$ ,  $P = 0.942$ ; effect of population as a fixed factor :  $F_{7,204} = 8.220$ ,  $P < 0.0001$ ): within population, the egg masses with higher HATCH % were not those with higher G25%.

Average HATCH% of population and average G25% did not correlate with census size of populations ( $r = 0.070$  and  $r = 0.299$  respectively,  $N = 8$ , all  $P > 0.47$ ): the larger populations were not those with higher survival.

Out of 590 non hatched eggs, in isolated populations  $48.5 \pm 7.9\%$  started larval development but died prior to hatch, while 51.5% did not start embryo development. In non isolated populations,  $26.4 \pm 5.9\%$  started larval development but died prior to hatch, while 73.6% did not start embryo development. The proportion of eggs per clutch that started larval development but died prior to hatch was higher in isolated populations (ANOVA:  $F_{1,23} = 4.906$ ,  $P = 0.037$ ).

### Experiment 2

Average survival during growth was 94-100% in isolated populations and 86-96% in non isolated populations (Fig. 4a). Differences in survival during growth among isolated and non isolated populations were not significant ( $U_{10,13} = 55$ ,  $P = 0.390$ ). Average survival at metamorphosis was 76-90% in isolated populations and 91-94% in non isolated populations (Fig. 4b). Average survival was always lower in isolated populations (Fig. 4b); however, these differences were not significant ( $U_{10,13} = 49$ ,  $P = 0.273$ ), possibly due to the low number of investigated populations.

### Summary of results

If average data from different populations are pooled, average ( $\pm$  SE) HATCH% was  $46.6 \pm 4.8\%$  in isolated populations ( $N=3$ ) and  $86.7 \pm 3.8\%$  in non isolated populations ( $N = 5$ ); G25% was  $52.7 \pm 8.4\%$  in isolated ( $N=3$ ) and  $92.0 \pm 1.8\%$  in isolated populations ( $N=5$ ); survival during growth was  $97.3 \pm 1.8\%$  ( $N=3$ ) in isolated populations and  $91.0 \pm 5.0\%$  ( $N=2$ ) in non isolated populations; survival during metamorphic climax was  $85.0 \pm 4.5\%$  in isolated populations ( $N=3$ ) and  $92.5 \pm 1.5$  in non isolated populations ( $N=2$ ). The product of these four

survival percentage during four different developmental stages is 20.3% for isolated populations and 67.1% in non isolated populations (Fig. 5). Thus, in absence of external factors of mortality such as predation or desiccation, only 20.3% of tadpoles of isolated populations survive until the metamorphosis; the survival of non isolated populations is more than three times higher.

## Discussion

Our results clearly show that individuals from isolated populations suffer reduced survival during larval stages, if compared with those from conspecific non isolated populations. The lower HATCH% in isolated populations is rather caused by an increase in the percentage of embryos dying during earlier development stages, than by an increase in the percentage of not fecundated eggs: the analysis of 590 non hatched eggs showed that in isolated populations there is an increased frequency of eggs that died after starting embryo development. Similarly, larvae from isolated populations showed a low survival also in the period immediately after hatch. Since survival strongly affect fitness, these results strongly suggest a loss of fitness in isolated populations. In turn, it is reasonable to hypothesize that this loss of fitness is caused by inbreeding depression (*sensu* Keller and Waller, 2002).

However, despite the strong differences among isolated and non isolated populations in survival at earlier developmental stages, differences were not significant in the experiment 2, investigating the survival after the developmental stage 25. Different explanations can be hypothesized for this discrepancy between experiments. First of all, in experiment 1 the sample size (218 egg masses, 7335 embryos) was one order of magnitude higher than in the experiment 2 (23 egg masses, 230 tadpoles), resulting in a much higher a-priori power and an higher possibility to detect significant differences (Steidl *et al.*, 1997). Moreover, in experiment 2 tadpoles were reared in a laboratory with constant 20°C, while in experiment 1 eggs were

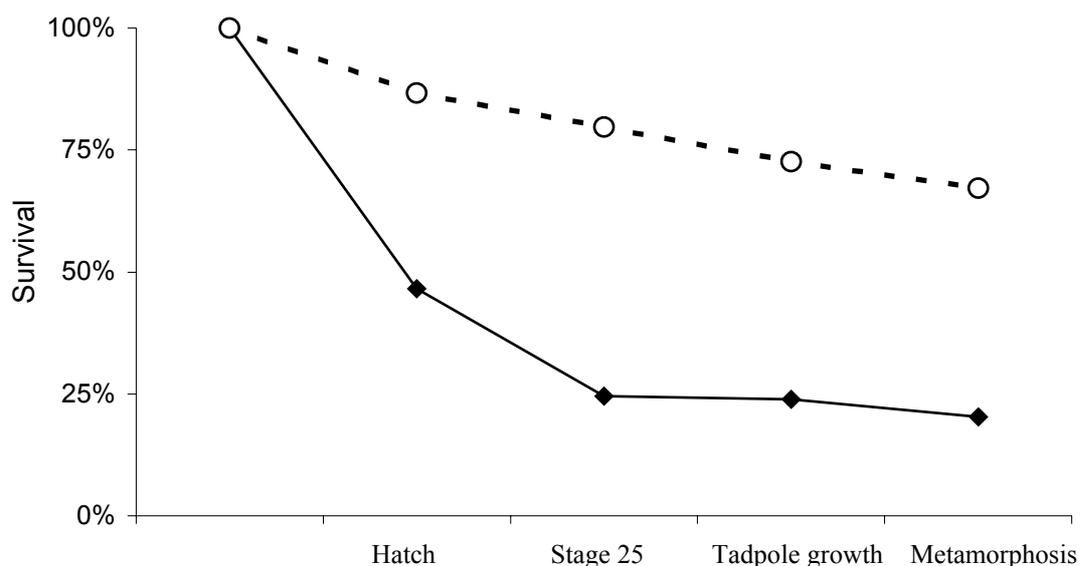


Fig. 5. Cumulative survival from hatch to metamorphosis of individuals from isolated (black diamonds) and non isolated (empty circles) populations, assuming 100% fertility of eggs.

maintained at outdoor temperature, in March. Early development stage are very sensitive to every kind of stress: in March, daily oscillations for temperature are strong, thus, it is possible that in experiment 1 conditions were more stressful than in experiment 2. In turn, stressful environmental conditions could have magnified inbreeding depression (Wright, 1922; Bijlsma *et al.*, 1999). It is possible that the small size of experimental container (0.2 l) exacerbated the role of thermal oscillations, since in container holding less water thermal oscillation are more pronounced. We were forced to use small container, due to the limited laboratory room. Finally, it is possible that inbreeding depression is more evident in earlier developmental stages, since we expect major genes to be expressed early in development (Keller and Waller 2002).

It could be hypothesized that the dramatic mortality observed in isolated populations were caused by factors other than inbreeding depression, such as contamination and diseases, or that rearing conditions were particularly unfavourable to isolated populations, thus reducing their survival. However, despite our study populations live in slightly different climates, differences among sites in average temperatures are 1.5°C or less (Belloni and Annovazzi, 1999; see also Ficetola and De Bernardi 2005). The laboratory was close to the sites were the populations MZ and CU breed: thus, environmental conditions were more similar to those encountered in nature by these two populations and in principle more suitable for them. However, these isolated populations were among those suffering lower survival. Contamination and diseases are unlikely because all clutches were randomly sorted in the same environment and in containers that were not been used before this experiment: if experimental contamination would occur, it would affect clutches from every populations. Moreover, during field surveys performed during spring 2003, in May we observed residuals of not hatched *R. latastei* egg masses in the wetland where population AL breeds, showing that also in nature many eggs did not hatch. We performed surveys also in the wetland were the population A2 breeds (Ficetola and De Bernardi, 2005), however, we did not observe residuals of not hatched egg masses in this wetland. Finally, in 2004 we collected for a different experiment clutches from some of our study populations, and we reared them in different conditions: also during this experiment, populations MZ and CU showed a reduced HATCH% if compared with populations T2 and A2 (G.F. Ficetola, unpublished data). The constancy of reduced survival across years and across populations strongly suggest that the reduced survival commonly occurs in these populations.

The inbreeding depression we observed strongly affected the survival of embryos: natural selection is thus expected to have a strong effect in purging deleterious mutations causing the early mortality of individuals (Waller, 1993). Our populations are likely to be completely isolated, and isolation is documented to last since long time at least in the population MZ: hence, it is not clear why did strong inbreeding depression persist, and the purging effect did not mitigate their effects. Purging can be less effective if genetic mechanisms such as overdominance mechanisms are present (Keller and Waller, 2002), or if inbreeding depression interact with environmental features (Bijlsma *et al.* 1999; Reed *et al.*, 2002).

Indeed, it is possible that purging occurs in our populations: despite isolation still persist, further inbreeding and drift do not causes a further reduction of fitness. The presence of purging could avoid that a population overcome a threshold of mortality, and could explain why do population MZ has a similar survival than do populations AL and CU, despite population MZ is likely isolated since a longer time. Moreover, a further reduction of fitness could drive the

populations in a mutational meltdown, thus causing quickly their extinction: observations of populations in the verge of a mutational meltdown are scarce, likely because these populations go extinct quickly (Rowe and Beebe, 2003).

The populations with lower HATCH% were those with lower G25% (Fig. 2): this result suggest that a given population showed similar inbreeding depression for different traits. However, after keeping into account differences among populations, the relationship between HATCH% and G25% was not significant. We hypothesize that mortality during earlier developmental stage could be caused by the presence of lethal mutants in genes expressed at these stages. Genes involved in the pre-hatch stages of embryo development (development stages 1-18) are different from those expressed in the post hatch stages (18-25): the phenotypic correlation between traits related to different genes, in absence of linkage disequilibrium, is expected to be zero, a result similar to those we obtained. From hatch to stage 25, strong modifications of tadpoles occur: for example external gills disappear, and the mouth parts are formed. These features are vital for free swimming tadpoles, and were not expressed earlier in the onthogeny (McDiarmid and Altig, 1999). Thus, our results support the hypothesis that, within population, inbreeding depression is affecting different genes, and these genes are independent; moreover, among populations, inbreeding depression similarly affects different genes.

We showed that, under the same environmental conditions, mortality in tadpoles from isolated populations is more than three times those observed in tadpoles from non isolated populations. Thus, populations having a reduced fitness are expected to face off a great risk of extinction. However, at least one population (MZ) is surviving in isolation since 1805. Generation time of *R. latastei* is about one year (Guarino *et al.*, 2003; Guarino and Mazzotti, 2004), thus the isolation lasted 150-200 generations. How did this population survived in complete isolation, if the population suffer strong depression? First of all, each *R. latastei* female can lay more than 1000 eggs per clutch (Bernini *et al.*, 2004b). If only 20% of each clutch survive, 200 tadpoles of each clutch survive. Survival of tadpoles is a process density dependent (Dash and Hota, 1980; Vonesh and De la Cruz, 2002). Indeed, the number of metamorphs arising from a wetland is not a linear function of number of tadpoles starting development in that wetland. In anurans, a lower number of tadpoles can produce a larger number of metamorphs, if the reduced density causes a reduced mortality (Vonsesh and De la Cruz, 2002). Thus, the reduced viability of each clutch may have not negative consequences on the survival of populations, since the death of some tadpoles can enhance the survival of siblings. Moreover, if natural selection purge the inbred tadpoles, the large number of offspring produced by each female allow a relatively large number tadpole to survive. It is possible that surviving tadpoles are the less inbred ones, with a fitness higher than the average of their population. These individuals could be fit also at later development stages, and survive until the reproduction. Indeed, our data at least partially support these considerations, since the tadpoles from isolated populations did not have significantly poorer performance during larval growth and metamorphosis, if compared with tadpoles from non isolated populations (Fig. 4). These considerations could be very important for understanding the evolutionary dynamics of isolated populations, and to manage them: further studies are needed to assess their consistence.

We observed a dramatic reduction of fitness in individuals from isolated populations of *R. latastei*. An alarmist point of view would suggest immediate plans for their management. A management tool suggested to increase genetic diversity of small, isolated populations is supplementation: in supplementation, individuals from different populations are translocated where the populations affected by inbreeding depression live. These actions can increase the genetic diversity and mitigate the inbreeding depression (reviewed by Hedrick, 2001). However, we think that translocations should not be performed with our populations. The conservation of intraspecific genetic diversity is a central conservation problem (Huges *et al.*, 1997), and the potential of a species evolutionary success is maximised through the maintenance of adaptive diversity (Frankel, 1974; Hedrick, 2001). However, we showed that different *R. latastei* populations can be considered significant evolutionary units, thus mixing different populations could indeed have negative on their fitness (Ficetola and De Bernardi, 2005: see chapter 9). In frogs with fast generation time, local adaptation can occur also in a few generations and among populations that are only a small geographical distance away (Skelly and Freidenburg, 2000; Skelly, 2004). Thus, translocation should be carefully considered, and populations suspected to be evolutionary significant units should be managed independently (Crandall *et al.*, 2000). Indeed, despite translocation is a relatively cheap and fast management tool, allowing a fast genetic recovery, we do not think that it can be useful for the long term persistence of populations also despite different evolutionary units were not mixed. If after translocation the isolation persist, in some generation inbreeding depression would occurs again, and the survival of populations could be again threatened. The positive effects of translocation would be lost in a few years: different management plans should be considered. For example, habitat management could be performed, to increase the habitat availability, and thus to allow a natural demographic recovery. If multiple, suitable wetlands are created or restored close to those occupied by the isolated populations, these wetlands can be colonised and occupied by new local populations, thus creating a metapopulation. In turn, a metapopulation can better retain the genetic diversity than do a single population with equal census size (Van Oosterthout *et al.*, 2004). Creating new wetlands thus increases both the census size and the effective population size, enhancing the effectiveness of natural selection and mutation in the natural increase of genetic diversity. Moreover, within a metapopulation the extinction / recolonisation dynamics could occur, allowing the long term survival of populations (Hanski and Gilpin, 1997). If metapopulations are established, the possibility of long term survival and the increase of the effective population size could allow, in a long term perspective, the natural increase of genetic variation.

We are aware that plans involving the habitat management and the increases of connectivity f landscapes are more expensive and would take long time than do translocations, however, we think that they would provide the better perspectives for the long term survival of populations.



## 8. SUPPLEMENTATION OR IN SITU CONSERVATION? EVIDENCE OF LOCAL ADAPTATION IN THE ITALIAN AGILE FROG *RANA LATASTEI* AND CONSEQUENCES FOR THE MANAGEMENT OF POPULATIONS

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*Animal Conservation*, 8: 33-40.

### **Abstract**

Relocation of endangered species can be an effective conservation tool if it does not mix populations that represent significant intraspecific variation. The threatened Italian agile frog, *Rana latastei*, has small populations with low genetic diversity: translocation has been proposed to improve the likelihood of survival of populations. Using a common environment experiment and field surveys, we investigated whether there were differences in larval growth and development rate between foothill and lowland *R. latastei* populations, to evaluate if they are evolutionary significant units. In nature, the colder climate of the foothills causes delayed metamorphosis. Conversely, in a common environment larvae from foothill populations show faster growth and development. We did not find a significant egg-size related maternal effect, or any differences in size at metamorphosis. We hypothesize that countergradient selection promoted fast growing phenotypes in a cold environment, where low temperature slow down larval development. Foothill populations, despite being only a small geographic distance away from lowland populations, seem to be adapted to a colder climate and represent a significant evolutionary unit. Different populations should, therefore, be managed independently, avoiding translocation. We suggest that evolutionary divergence between populations should be verified prior to planning relocation programs, to prevent the risk of genetic homogenisation.

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### **Introduction**

Relocation of animals is a popular management tool for the conservation of threatened species. For example, in past decades there have been many attempts to re-introduce species to areas from which they had become extinct, with successful results, at least in some cases (reviewed in Griffith *et al.*, 1989; Fischer and Lyndenmayer, 2000). Supplementation is another relocation action frequently used in conservation biology (Storfer, 1999). In supplementation, individuals are added to an existing population of conspecifics. If the source population is wild ranging, the movement of animals is called translocation (for definitions, see IUCN, 1995).

In supplementation actions, individuals can be moved in areas when habitat deterioration or hunting has caused a decreasing population size. Supplementation could reduce the vulnerability of recipient populations to environmental and demographic stochasticity, allowing faster population growth and the recolonization of newly suitable habitat (Lubow, 1996). Moreover,

supplementation has been proposed as a tool to manage populations that are facing the problems of inbreeding depression (Hedrick, 2001). Such small, isolated populations can be threatened by loss of genetic diversity caused by genetic drift, by the fixation of deleterious mutations caused by the low efficiency of natural selection and by increased inbreeding. Since these phenomena, together, can cause loss of fitness (see Keller and Waller, 2002), introducing individuals from populations with higher genetic diversity potentially could restore the genetic diversity and fitness of small, isolated populations.

However, supplementation could also have detrimental effects on endangered species, because it leads to a reduction of genetic differences between populations (Moritz, 2002). Intraspecific genetic diversity is recognised as a central conservation problem (Huges *et al.*, 1997; Sinclair, 2001), and the potential for a species' evolutionary success is maximized through the maintenance of adaptive diversity (Frankel, 1974; Hedrick, 2001). Populations representing significant adaptive variation are not ecologically exchangeable (Crandall *et al.*, 2000): several authors define them as "evolutionary significant units", suggesting that they should be independently managed, to avoid genetic homogenisation (Ryder, 1986; McKay and Latta, 2002; but see also Moritz, 2002). Evaluating whether different populations are evolutionary independent is, therefore, an important question to resolve prior to supplementation. Nevertheless, experimental tests of adaptive divergence between population are scarce in endangered species (Hedrick, 2001).

Among vertebrates, amphibians are considered by some authors to be potential candidates for relocation programs (Marsh and Threnham 2001; Semlitsch 2002; but see Seigel and Dodd, 2002). Since each female produces many eggs, it is relatively easy to rear them and to have large stocks for reintroduction or supplementation: therefore, several projects of relocation of threatened amphibians have been carried out and/or are ongoing worldwide (see Burke, 1991; Semlitsch, 2002). The Italian agile frog, *Rana latastei*, is a brown frog that is endemic to the lowlands of northern Italy (Padano-Venetian plain) and adjacent countries. It lives in riparian woodlands, breeding in river washes and ponds (Pozzi, 1980). These lowlands are currently dominated by agriculture and urbanization and now *R. latastei* survives only in a small number of frequently small and isolated woodlands. Moreover, the isolation of suitable patches has increased the probability of extinction of these populations (Ficetola and De Bernardi, 2004). Fewer than 100 breeding sites have been recorded for this species in the European herpetological atlas; therefore *R. latastei* is considered to be one of the most threatened amphibians in Europe (Grossembacher, 1997) and is rigorously protected in the European Union (EC 43/1992). Plans are ongoing for its conservation (Gentilli *et al.*, 2003).

Recent studies have revealed a loss of genetic diversity in peripheral populations in the north-western edge of *R. latastei*'s range, when compared to populations with a more central or eastern range (Garner *et al.*, 2003, 2004a). Moreover, preliminary data suggest that some peripheral populations that are isolated and on the northern edge of the range, could have a lower survival than populations with a more central location (Ficetola *et al.*, 2003, see also chapter 7). Translocation of individuals from southern to northern populations has been, therefore, proposed to increase genetic diversity and to avoid the risk of inbreeding depression (Garner *et al.* 2003).

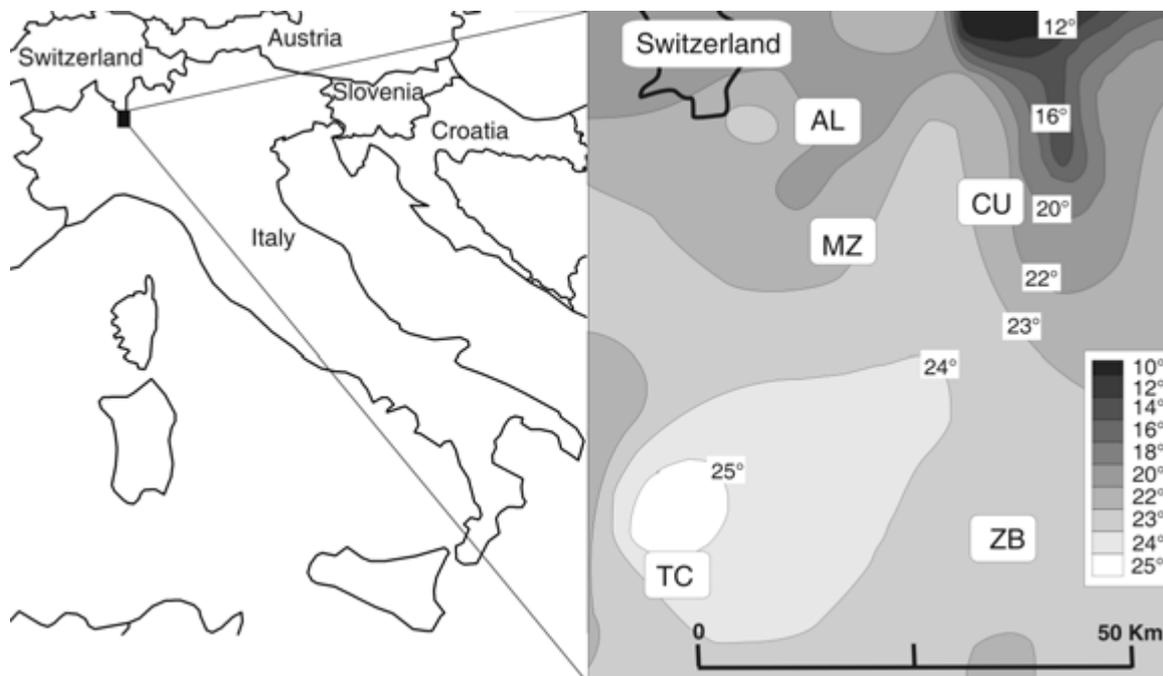
We investigated differences in larval performance between foothill and lowland populations of *R. latastei* of Lombardy (Northern Italy), to evaluate whether they belonged to different

evolutionary units. Foothill populations are small in size and are on the northern edge of the species distribution. Conversely, the southern lowland populations that we studied are larger and live along rivers. The geographic distance between populations is small (< 60 Km): hence the lowland populations could be good sources of individuals for translocation to increase genetic diversity of foothill populations (Garner *et al.*, 2003). However, it is also possible that these populations evolved in slightly different climates or habitats. Prior to make translocation plans, it is important to evaluate whether the populations have heritable differences caused by evolutionary processes. If differences exist, the populations should be considered evolutionary independent units, and they should be protected *per se*, avoiding translocation; *in situ* conservation would be more appropriate. We measured larval life history traits (growth and development rates, size at metamorphosis) since these can evolve in relation to climatic differences (Conover and Shultz, 1995) and are important traits affecting fitness of anurans (Semlitsch, 2002 and references therein). We compared larval performance of hill and lowland populations in nature and under common laboratory conditions. We also measured maternal effects (see Methods, below) to evaluate the differences between populations excluding potential environmental effects.

## Methods

### *Study area and population sampling*

We sampled five localities in Northern Italy where *R. latastei* populations live (Fig. 1). These populations have different geographic setting: three are in the hills of Brianza (AL, CU and MZ)



**Fig. 1.** Location of sampled populations (Lombardy Region, Northern Italy), and average daily temperature (°C) in July. Temperature map was redrawn from Belloni and Annovazzi (1999). TC and ZB are lowland sites, AL, CU and MZ are foothill sites.

at altitudes of 320, 300 and 175 m above sea level (a.s.l.), respectively; two populations (TC and ZB) are found in River Po lowlands at altitudes of 69 and 76 m a.s.l. respectively. The hill populations are on the northernmost range edge and near to the altitudinal limit of *R. latastei* distributions (Grossenbacher, 1997). The number of breeding females, as estimated by the number of egg masses, was 43, 10 and 150 respectively in the populations AL, CU and MZ, and 300 and 1000 respectively in the populations TC and ZB. All of these populations breed in wetlands in or very near to hornbeam and pedunculate oak woods and are protected in Natural Parks. During early march 2003, we gently removed from each population a small fraction ( $58 \pm 5$  eggs) of five spawns laid during the night before the sampling day. We transferred the eggs in 200 ml plastic container and we returned them the day after to the laboratory.

#### *Larval performance measures in the field*

To evaluate differences in age at metamorphosis between populations in the field we dip-netted one hill population (AL) and one lowland population (ZB). During each dip-netting session we captured and briefly examined at least 50 tadpoles from the different microhabitats of these two sites, in order to evaluate their development stage. All of the tadpoles were liberated immediately after examination. This dip-netting was carried out every 2nd 4th day from May 2003 until the first metamorph was found (Gosner (1960) developmental stage 42: emergence of forelimbs). Only two sites were examined, since at the sites CU and TC, *R. latastei* is syntopic with *R. dalmatina*: the tadpoles of these two species are similar and it is not possible to unambiguously distinguish between them (e.g., Vercesi *et al.*, 2000). At the site MZ an anthropogenic pond drying killed all tadpoles prior to the metamorphosis.

#### *Larval performance measures in laboratory*

We reared spawn samples under standard laboratory conditions (constant 20°C with 12-h light-dark cycles). Immediately after hatching, 10 tadpoles were randomly selected from each clutch and put in the same plastic container filled with 1.5 l of aged tap water. Thus, the experimental design was 10 tadpoles per clutch, 5 clutches per population, 5 populations, giving a total of 250 tadpoles. The containers were randomly sorted over the same bench in the laboratory. After reaching Gosner's stage 25, the tadpoles were fed *ad libitum* with rabbit chow and lettuce and water was changed weekly.

The tadpoles were weighed to the nearest 0.01 g on reaching stage 25 in order to evaluate the tadpole starting weight. They were weighed again 24, 31, 37 and 45 days after hatching. During weighing, each tadpole was individually removed from the water, carefully blotted dried and weighed; the tadpole was then immediately returned in its container. Age in days from hatching until the metamorphosis (defined as the almost complete tail resorption (Gosner's stage 45)), was recorded and metamorphs were weighed to the nearest 0.001 g. After the metamorphosis all of the froglets were liberated in their wetland of origin.

Tadpole weight 37 days after the hatching was taken as a measure of the tadpole growth in terms of weight gain rate, since soon after the fastest developing tadpoles began metamorphic

climax and lost weight (Fig. 2). We considered that the age at metamorphosis was a measure of development rate.

### *Statistical analysis*

In all our analysis, we used the average value for the tadpoles from each container for each larval performance measure (starting weight, weight during growth, age at metamorphosis, weight at metamorphosis), since the tadpoles in the same container were siblings. Towards the end of the experiment, a few tadpoles died in some containers resulting in reduced densities in some of the replicates. Therefore we added the final density of each tadpole family (DENSITY) as covariate in our analysis.

We used mixed model analysis of variance to evaluate the effects of geographic position (GEOG: hill/lowland), population of origin (POP), tadpole starting weight (W\_START) and final density (DENSITY) on larval performance features of *R. latastei* tadpoles. We used GEOG as a factor and considered POP nested within GEOG as a random factor; we used W\_START as covariate to check for maternal effects (see Parichy and Kaplan, 1992; Zeisset and Beebee 2003 for a similar approach), since tadpole hatching size is strongly correlated with egg size (Kaplan, 1998; Laugen *et al.*, 2003b). As dependent variables, we used the weight 37 days after hatching (W\_37D), age at metamorphosis (AGE\_METAM) and weight at metamorphosis (W\_METAM). The residuals were normally distributed. All means are  $\pm 1$  standard error.

## **Results**

### *Field study*

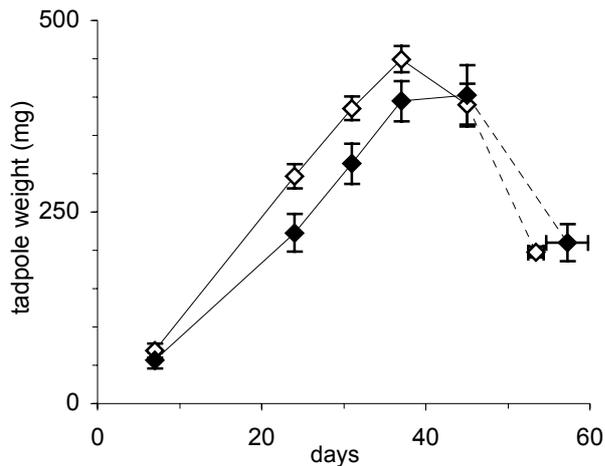
All of the populations laid eggs approximately in the same period (March 1-15). We caught the first metamorph in the lowland site ZB on May 17. During the same day, the most developed tadpoles we caught in the hill site AL were at the developmental stage 33 (toe development of hind limbs). We caught the first metamorph in the hill site AL on June 18. During the same day, we did not find any *R. latastei* tadpoles in the lowland site ZB, probably because they had all metamorphosed prior to this date. Therefore, in the field, AL (hill) tadpoles appeared to take approximately a month longer to complete the metamorphosis than did ZB (lowland) tadpoles.

### *Laboratory study*

None of the embryos from two spawns samples of the population CU survived until the stage 25, hypothetically for inbreeding depression, therefore we dropped these two spawns from all our analyses. Overall, we considered tadpoles from 23 spawns: 13 from the foothill populations and 10 from the lowland populations.

At 20° C, the tadpoles from foothill populations grew faster and metamorphosed earlier than those from lowland populations (Fig. 2). Average weight 37 days after hatching was  $449 \pm 9$  mg

for foothill tadpoles, and  $395 \pm 13$  mg for lowland tadpoles (Fig. 3a). The first tadpoles

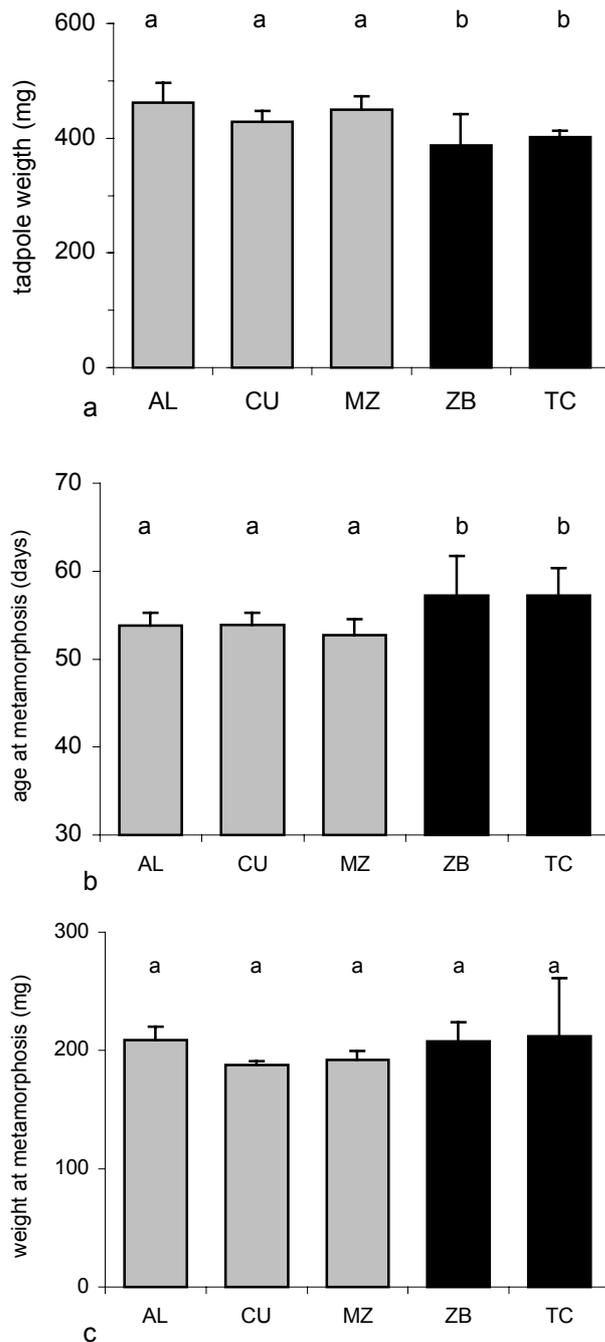


**Fig. 2.** Growth curves and weight at metamorphosis of tadpoles reared in a common environment. White diamonds: tadpoles from foothill populations; filled diamonds: tadpoles from lowland populations. Error bars = two standard errors; horizontal error bars = two standard errors of age at metamorphosis. Weight at metamorphosis was taken from animals already living in a subaerial environment (i.e. metamorphosis was complete and the froglets were now air-breathers).

metamorphosed 48 days after the hatching: they were from the population AL (hill); the last one metamorphosed 79 days after hatching: it was from the population ZB (lowland). On average, foothill tadpoles metamorphosed  $53.4 \pm 0.5$  days after hatching while lowland tadpoles metamorphosed  $57.2 \pm 1.3$  days after hatching: development rate was therefore 7.1 % faster in the foothill populations (Fig. 2; 3b). Weight 37 days after hatching and age at metamorphosis were strongly correlated ( $r = -0.694$ ,  $N = 23$ ,  $P < 0.001$ ): the tadpoles that grew faster after the hatching also metamorphosed earlier. The relationship between growth rate and weight at metamorphosis was not significant ( $r = 0.148$ ,  $N = 23$ ,  $P = 0.50$ ). We observed a tendency for the last metamorphosing tadpoles to be heavier than the first ( $r = 0.388$ ,  $N = 23$ ,  $P = 0.067$ ).

We observed a significant effect of geographic positions on larval performance, but differences were not present among populations within groups (Tab. 1). Tadpole starting weight did not have a significant effects on larval performances, suggesting lack of egg size related maternal effect. Tadpoles from foothill populations grew faster and metamorphosed earlier than tadpoles from lowland populations (Tab. 1, Fig. 3a, 3b). However, geographic position did not seems to influence the weight at metamorphosis (Tab. 1, Fig. 3c). Age at metamorphosis was not related to final density in experimental containers. However, the tadpoles from containers with lower density were the heaviest both 37 days after hatching and at metamorphosis (Tab. 1). Since we did not observe a significant variability within group for any measure of larval fitness, we repeated the analysis pooling data of populations with the same geographic position. The results are the same obtained using the complete model. Weight 37 days after hatching was significantly higher in foothill populations ( $F_{1,19} = 17.630$ ,  $P < 0.001$ ) but it did not seem to be affected by starting weight ( $F_{1,19} = 0.023$ ,  $P = 0.881$ ). Foothill populations metamorphosed significantly earlier than did lowland populations ( $F_{1,19} = 6.168$ ,  $P = 0.023$ ) but age at metamorphosis did not

seem to be affected by starting weight ( $F_{1,19} = 0.222$ ,  $P = 0.643$ ). Weight



**Fig. 3.** Larval performance of *R. latastei* tadpoles from five populations. Grey bars: foothill populations; black bars: lowland populations. error bars equal two standard errors. a: weight of tadpoles 37 days after hatching. b: age at metamorphosis. c: weight at metamorphosis. Different letters indicate significant differences for the larval performance measure considered.

at metamorphosis was not different between foothill and lowland populations ( $F_{1,19} = 0.0764$ ,  $P = 0.786$ ) nor did it seem to be affected by starting weight ( $F_{1,19} = 0.364$ ,  $P = 0.553$ ). Age at metamorphosis was not related to final density ( $F_{1,19} = 0.129$ ,  $P = 0.723$ ); however the tadpoles from lower density containers were the heaviest both 37 days after hatching ( $F_{1,19} = 5.012$ ,  $P = 0.037$ ) and at metamorphosis ( $F_{1,19} = 56.476$ ,  $P < 0.001$ ).

Factors	<i>F</i>	df	<i>P</i>
dependent: W_37D			
GEOG	20.152	1, 4.7	<b>0.007</b>
POP	0.508	3, 16	0.682
W_START	0.453	1, 16	0.511
DENSITY	4.730	1, 16	<b>0.045</b>
dependent: AGE_METAM			
GEOG	9.838	1, 6.1	<b>0.020</b>
POP	0.294	3, 16	0.829
W_START	0.702	1, 16	0.415
DENSITY	0.016	1, 16	0.902
dependent: W_METAM			
GEOG	0.241	1, 3.7	0.651
POP	1.168	3, 16	0.353
W_START	0.693	1, 16	0.417
DENSITY	55.521	1, 16	<b>&lt;0.001</b>

**Table 1.** Effects of geographic position (GEOG), population (POP), starting weight (W\_START) and final density in experimental containers (DENSITY) on *R. latastei* tadpoles weight 37 days after hatching (W\_37D), age at metamorphosis (AGE\_METAM) and weight at metamorphosis (W\_METAM).

## Discussion

### *Evolutionary meaning of between-population differences*

Under the same laboratory conditions, foothill populations of *R. latastei* showed significant differences from lowland populations in larval performance traits. Ignoring potential maternal effects, our results seem to have an unambiguous interpretation: foothill populations have a capacity for faster growth and development and this differentiation is difficult to interpret other than in the light of local adaptation to some selective factor (Merila *et al.*, 2000; Laugen *et al.*, 2003a). Egg size is a major source of maternal effects in amphibians (Kaplan, 1998). Starting weight did not significantly affect larval performance, thus, we suggest that differences in larval performances between these populations are mainly caused by genetic differentiation and not because of egg-size related maternal effect (cf. Laugen *et al.*, 2003a).

Many studies have demonstrated that growth and development rates strongly influence the fitness of amphibians. High growth and development rate enable tadpoles to metamorphose quickly to escape death in drying ephemeral ponds, escape aquatic predators, or to maximise size at metamorphosis, therefore these traits should be subject to strong natural selection (Wilbur and Collins, 1973; Travis *et al.*, 1985; Banks and Beebe, 1988; Newman, 1988a; 1988b). Several factors could have caused the observed differences between populations: among them, the effect of temperature on the selection of a fast-growing phenotype seems to be the most likely.

Water temperature strongly affect development and growth rate in amphibians: tadpoles living in a colder environment grow and develop slowly. A few studies outlined that when compared in a common environment, larvae from populations living in colder areas showed the genetic capacity to complete metamorphosis faster (Berven *et al.*, 1979; Berven, 1982a; Loman,

2002a). When geographic variation in genotypes is in opposition to environmental influence, a trait displays countergradient variation (Conover and Shultz, 1995). Our results are consistent with this pattern and could be a new case of countergradient variation in amphibians. In our study case, foothill populations of *Rana latastei* live in a slightly colder climate, with average spring and summer temperature 1-2°C lower than that for lowland populations (Belloni and Annovazzi, 1999; Fig. 1). Therefore, in nature, the tadpoles of foothill populations metamorphosed later than did the tadpoles from lowland populations. However, in a common environment the foothill populations showed the capacity to grow and metamorphose earlier. Thus, it is likely that natural selection favoured a fast-growing genotype in the foothill populations, living in a colder climate. Individuals not adapted to the hill environment are expected to have a slow growth and development rate if reared in the colder climate of hills and, therefore, to have a too delayed metamorphosis (Berven *et al.*, 1979).

Our study dealt with only relatively few sites, tadpoles were reared at only one temperature, and the populations can not be placed along a continuous climatic gradient. Therefore, it is possible that the differences seen in larval performance are caused by forces other than countergradient selection. For example, it is possible that the populations are adapted to different thermal optima: tadpoles develop relatively faster when they are reared at temperature more similar to those of their wetland of origin (Skelly and Freidenburg, 2000). Moreover, there is some evidence that frog populations living in temporary ponds can evolve a faster development rate or a higher developmental plasticity, to reduce the risk of desiccation (Wilbur and Collins, 1973; Laurila *et al.*, 2002; Loman 2002b). However, none of the wetlands of our study was temporary, therefore this latter hypothesis seems to be unlikely. The desiccation of the site MZ was an unusual event, caused by the inappropriate maintenance of a pond, since this population resides in a urban park. Finally, other differences among sites can not be ruled out, such as differences in predator abundance (Lardner, 1998), differences in food availability or other unrecognised differences among habitats.

#### *Local adaptation: implications for the management*

We observed variation that could be adaptive between populations of *R. latastei* living in sites quite close together and with rather small ecological differences. Thus, foothill and lowland populations are ecologically distinct and there is not exchangeability between them (Crandall *et al.*, 2000): the lowland populations should not be used as source of individuals in supplementation projects aimed to increase size and/or genetic diversity of the foothill populations. Indeed, translocation could have negative effects for the conservation of *R. latastei*. First of all, the genetic homogenisation could undermine the evolutionary potential of the species. In a human-dominated environment, with fast and unpredictable modifications, the evolutionary potential has pivotal importance for the persistence of the species. Moreover, lowland individuals introduced to a hill site could have very low fitness (home-site advantage), since they may be poorly adapted to the new environment, suffering unusually high mortality or lowered reproductive success (Montalvo and Ellstrand, 2000). For example, translocated tadpoles could have delayed metamorphosis or higher mortality (Berven *et al.*, 1979). Similarly, foothill tadpoles transplanted to the lowland environment would be not adapted to the warmer

climate, suffering a reduced ability to cope with higher temperatures (Skelly and Freidenburg, 2000). Furthermore, it is possible that populations differ for other life history traits not investigated in this study, like size and age at first reproduction (Berven, 1982b), and, therefore, the home-site advantage could be even stronger. Finally, mixing different populations could result in a reduced fitness of hybrids, a phenomenon called outbreeding depression (Storfer, 1999; Montalvo and Ellstrand, 2000; Edmands and Timmerman, 2003).

The foothill populations are small and isolated, and they face a high risk of extinction due to environmental and demographic stochasticity and due to potential inbreeding depression. Since these populations seem to represent significant adaptive variation for *R. latastei*, their protection should be prioritised for the conservation of this threatened frog. Translocation projects are not acceptable, thus different management proposals should be made, emphasising *in situ* conservation. First, we should consider habitat management, such as the creation of new ponds, improvement in the suitability of existing wetlands and management of terrestrial habitat (Semlitsch, 2002). The availability of larger patches and of a more suitable habitat can allow the increase in the population size, however the results of these actions will require a long time. During the winter 2001-2002, new wetlands were excavated where the population AL lives. To date, this population seems to have reacted well to these actions, since the number of breeding females grew from 19 during 2001 to 43 during 2003 (A. Gentilli, pers. com.). Obviously, an increase of genetic diversity will require longer time than the does demographic growth (Hedrick, 2001). Eventually, for a faster recovery of intra-population genetic diversity, translocation of individuals within the foothill population group could be considered, since variability within group seems to be low in our study (Table 1). These actions should be planned only after more detailed studies on the differences between these populations and, preferably, after molecular genetic studies.

### *Conclusion*

We showed that *R. latastei* populations less than 50-60 Km apart and living in similar environments have significant differences in possible adaptive traits. Therefore, management actions such as translocation could lead to loss of intraspecific genetic diversity and evolutionary potential and, eventually, to outbreeding depression. Local adaptation could be more frequent than previously suspected in animals with low mobility and fast generation time, resulting in significant adaptive differences being present between populations close or living in similar habitats. Many studies have used variation at neutral genetic markers to evaluate differences between populations prior to plan translocation as a management tool (for a review, see Crandall *et al.* 2000). However, the correlation between neutral and adaptive variation can be low, and the use of neutral markers only would not reveal the presence of variants representing important intraspecific diversity (for a discussion, see Crandall *et al.*, 2000; Montalvo and Ellstrand, 2000; Hedrick, 2001; Pearman, 2001; McKay and Latta, 2002 but see also Moritz 1999; 2002). Therefore, the analysis of evolutionary variation, as measured by life history traits, could be a useful tool for the development of a correct decisional pathway for the management and conservation of wild populations, for example in relocation plans.

## 9. TRADE-OFF BETWEEN LARVAL DEVELOPMENT RATE AND POST METAMORPHIC JUMPING PERFORMANCE IN THE FROG *RANA LATASTEI*: CONSEQUENCES FOR THE MAINTENANCE OF COUNTERGRADIENT VARIATION

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### Abstract

Fast development rate early in the ontogeny is believed to correlate positively with fitness. However, in several species the persistence of countergradient variation allow to hypothesize the existence of trade offs between development rate and other fitness related traits. We investigated if these trade offs could exist between pre- and post-metamorphic traits in an organism with complex life cycle. In laboratory, we measured whether the tadpoles of the frog *Rana latastei* with fast intrinsic development rate have a suboptimal post-metamorphic morphology, by comparing froglets from five populations. Thus, we evaluated the relationship among age at metamorphosis, hindlimb length and jumping performance of frogs grown in nature. In laboratory, froglets with fast intrinsic development had shorter absolute and shorter size-adjusted tibiofibulas. In nature, froglets from the lat metamorphosing population had longer absolute and size-adjusted tibiofibulas, and jumped longer leaps. The cost of fast development could be the shorter legs of early metamorphosing frogs, and their poor jumping performance. Thus, a fast intrinsic development rate could be not always positively related with lifetime fitness. In *R. latastei*, the decoupling between pre- and postmetamorphic traits is not complete, and trade offs could persist also across life history stages.

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### Introduction

A trait display countergradient variation when the geographic variation of genotypes is in opposition to environmental variation. For example, a number of studies observed that populations living in colder climates have the genetic capability to grow and develop faster than conspecifics living in warmer conditions, to counteract the negative effect of low temperature that slow down the development rate (Conover and Shultz, 1995). When individuals from populations adapted to different thermal regimes are reared in the same environment, the individual from the colder environment are the fastest developing. However, since their environment of origin is colder, in nature their development can take longer time than those of populations living in warmer climates. For many organisms, including amphibians and insects, development and growth rate are traits believed to have a strong, positive association with fitness (see Scriber and Slansky, 1981; Semlitsch, 2002 for references). For example, fast growth and development enable amphibian tadpoles to escape death in drying ephemeral ponds and to escape aquatic predators, considerably enhancing their survival (Travis *et al.* 1985; Banks and

Beebee, 1988; Newman 1988a, 1988b). Thus, we expect that natural selection favoured the fastest developing genotype in all the environments. Given these observations, in some case countergradient variation can constitute an enigma to evolutionary biology: it is not clear why do populations from warmer environments sometime develop at rates less than the physiological capacity demonstrated by conspecifics from colder environments (Conover and Shultz, 1995; Skelly, 2004).

A few previous studies hypothesized the existence of trade-offs to explain the persistence of countergradient variations: growth and development in juveniles may be negatively correlated with other fitness related traits, such as locomotion, feeding, the reproductive output as adults or the ability to withstand predators and environmental changes (Conover and Shultz, 1995; Arendt *et al.*, 2001; Skelly, 2004). For example, the populations with high intrinsic growth rate of the fish *Lepomis gibbosus* have lower swimming speed and higher vulnerability to predation than do slow growing populations (Billerbeck *et al.*, 2001; Lankford *et al.*, 2001). More generally, there is evidence that individual growth rate is optimised to local conditions rather than maximised to be as fast as possible (Arendt, 1997; 2003; Morgan and Metcalfe, 2001). However, the trade-offs associated to development rate remain largely unexplored (Arendt, 1997): identifying the costs of rapid development is therefore an important next step for life history theory. Moreover, in organisms with complex life cycles, it is not clear whether the intrinsic development rate during earlier stages can have a delayed cost after the metamorphosis. For these organisms, the metamorphosis can allow the independent evolution of different life history stages, thus maximising the overall fitness of individuals (Moran, 1994). However, the decoupling between stages may be not complete, because of the persistence of developmental constraints (Watkins, 2001). Therefore, a fast growth rate early in the ontogeny may in principle have fitness costs that are not exhibited until a later developmental stage, thus making it difficult to detect the costs and benefits associated with the fast development rate. Amphibians are ideal candidates to explore this hypothesis. Countergradient variation for development rate has been demonstrated for several species (Berven *et al.*, 1979; Berven, 1982a, 1982b; Loman, 2002b; Laugen *et al.*, 2003; Skelly, 2004). Moreover, the environment that amphibians encounter during the larval development can influence not only the larval growth and development, but also the features of froglets after the metamorphosis, like morphology, survival and jumping performance (Blouin and Brown, 2000; Relyea, 2001; Alvarez and Nicieza, 2002; Relyea and Hoverman, 2003).

Here we test whether a fast larval development can have a cost after the metamorphosis in the Italian agile frog *Rana latastei*. *Rana latastei* is a rare species of brown frog endemic of Northern Italy and adjacent countries. It breeds in wetlands within lowland forest; the adults are terrestrial with the only exception of a few days during the breeding season (Pozzi, 1980; Ficetola and De Bernardi 2004). This frog has limited genetic variability and distributional range (Garner *et al.*, 2004a); however, if reared in the same controlled environment, individuals from foothill populations develop ~7% faster than do those from lowland populations: they live in a climate with average spring temperature 1-2°C colder, and thus seem to be adapted to this environment. Conversely, in natural conditions the colder environment causes delayed metamorphosis of foothill populations (Ficetola and De Bernardi, 2005). To evaluate if countergradient variation on larval development rate could indirectly affect the frogs also after the metamorphosis, we analysed the relationships between development rate, morphology and

jumping ability. These traits are considered to be important for the survival prior and after the metamorphosis (Wassersug and Sperry, 1977; Newman, 1988b; Henein and Hammond, 1997), and we hypothesized that froglets that develop more slowly can invest more resources in morphological traits that favour terrestrial locomotion. The cost of short larval period can be a suboptimal morphology in the post-metamorphic stage, resulting in decreased locomotor performance (Alvarez and Nicieza, 2002). First, we studied froglets reared under laboratory conditions to evaluate whether intrinsic differences in development rate are related to differences in post-metamorphic morphological traits. We evaluated these relationships both among siblings and among populations. Second, we compared froglets metamorphosed in nature from two different populations. These populations have different intrinsic development rate: the population with the fastest intrinsic development rate live in the colder environment, and in nature it develop more slowly. By comparing these two populations, we explored if differences in the age at metamorphosis cause differences in morphology comparable to those measured in laboratory. For these populations we also measured the jumping performance of froglets, to evaluate if the morphological differences can have a direct effect on fitness.

## Methods

### *Common laboratory experiment*

In spring 2003, we collected 23 recently laid clutches from five populations of *R. latastei* in Northern Italy (three foothill populations: AL, CU, MZ; two lowland populations: TC, ZB). We reared five clutches for each of the populations AL, MZ, TC, ZB; due to the low fertility of the clutches from site CU, we reared only three clutches from this population. See Ficetola and De Bernardi (2005) and the chapter 8 for further details on the study populations. After hatch, we haphazardly selected 10 tadpoles for each clutch and we put them in the same plastic container filled with 1.5 l of aged tap water. The 23 containers (total: 230 tadpoles) were randomly sorted over the same bench of the laboratory, at constant 20°C with 12-hour light-dark cycles. We fed tadpoles ad libitum with rabbit chow and lettuce; we changed water weekly. After the metamorphosis (Gosner's 1960 stage 45: almost complete tail resorption) froglets were weighed to the nearest 0.1 mg and photographed. Age at metamorphosis was recorded; we liberated all the froglets in their wetland of origin.

### *Performance of naturally grown froglets*

In spring 2004, we monitored one lowland population (ZB) and one foothill population (MZ). In late winter, we performed surveys each 2nd-7th day to detect the presence of egg masses. These sites were also periodically visited during spring, to verify the development of tadpoles. When tadpoles approached metamorphosis (late spring-early summer: see below), the populations ZB and MZ were intensively deep-netted to catch metamorphs (Gosner's stage 39-42). We restricted our sampling to these tadpoles since after stage 39 tadpoles lose feeding structures and stop growing in weigh (Gosner 1960). We avoided to catch later developmental stages, such as froglets living on the terrestrial environment, since it is possible that some of these animal started

feeding terrestrial invertebrates, and thus they would be not comparable with just metamorphosed froglets. We caught 13 metamorphs from the population ZB and 26 from the population MZ. We transferred metamorphs to the laboratory (constant 20°C) to standardize the environmental conditions, since differences in environmental temperature cause differences in jumping performance (e.g., Van Buskirk and Saxer, 2001): each metamorph spent a few days in the laboratory, i.e. the time elapsed from the sampling to the stage 45. At the Gosner's stage 45 froglets were weighed to the nearest 0.1 mg and photographed. To measure jumping performance, each froglet was put over a bench and covered by a Petri dish. When the cover was removed, usually froglet jumped, and we measured the length of the jump. For each froglet we measured three consecutive jumps, since after the 3rd – 4th jump some froglet was exhausted and did not jump anymore. We failed to obtain jumps from two froglets, thus, the number of jumping froglets was slightly lower than the number of measured froglets: overall, we obtained jumps from 13 froglets from the population ZB and from 24 froglets from the population MZ. Since tadpoles grew in the field, in completely natural conditions, it was not possible to assess the age at metamorphosis of froglets. However, in the population ZB, we observed the first clutches March 7, in the population MZ we observed the first clutch March 9. In both populations, most of clutches were laid within one week from the first ones, thus we assumed that at a given date the tadpoles of both sites had similar age.

#### *Data analysis*

For each froglet, we measured tibiofibula length on the basis of pictures; in our analysis we used the average of left and right tibiofibulas. In anurans, tibiofibula length is a trait strongly positively related with jumping performance (Zug 1972, see results). We used maximum jumping length as a measure of jumping performance, since maximum jumping length seems to be more strongly related to feeding and escape ability than average jumping length, and it shows high repeatability (Henein and Hammond 1997; Semlitsch *et al.*, 1999; Watkins 2001). Using average jumping length instead than maximum jumping would not change any of our results.

We used linear models to evaluate the relationships among population of origin, length of larval development, weight at metamorphosis, tibiofibula length and jumping performance. Because we were interested in both absolute and size-independent performance, we used both ANOVA and ANCOVA models, using body weight (thereafter weight) and tibiofibula length as covariates. For the froglets reared under laboratory conditions, we used mixed models, using geographic position of origin (lowland/foothill) as a fixed factor and clutch of origin as a random factor nested within geographic position. The complete model structure would include geographic position, population nested within geographic position and clutch within population within geographic position. However, population did not have a significant effect in any one of our models (all  $P > 0.4$ ), thus this factor was removed from the models for simplicity and to increase power (Sokal and Rohlf, 1995). Including population within any of our models would not change qualitatively any of our results.

For the froglets metamorphosed in nature, we do not know when each individual was born, thus we do not have data about the age at metamorphosis of each individual. However, all the froglets we caught from the locality ZB metamorphosed between June 7 and June 18, and all the

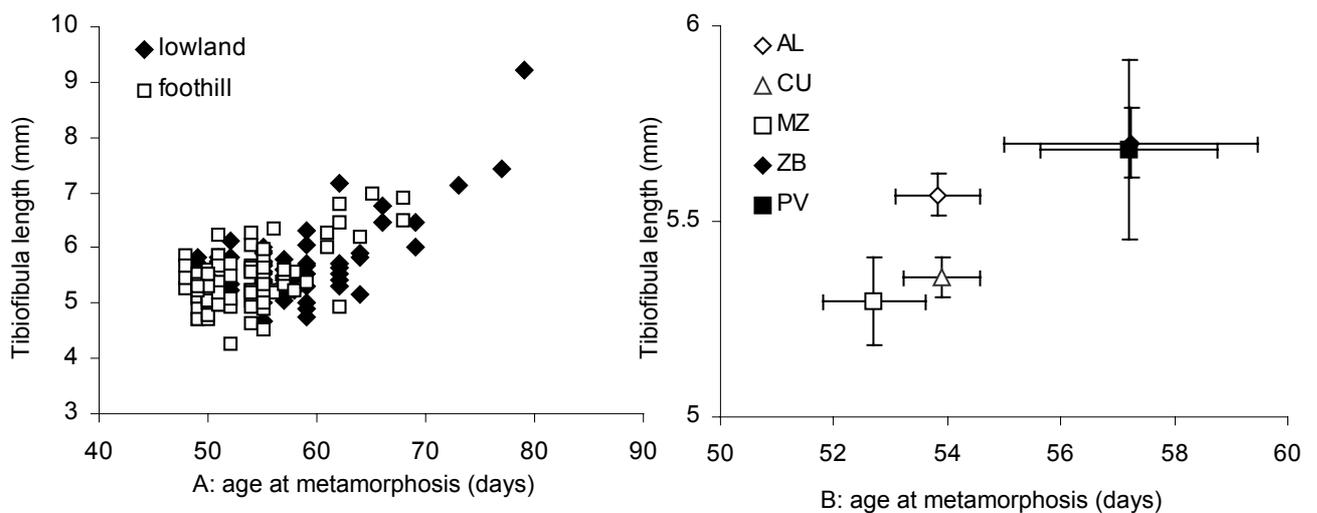
froglings from the locality MZ metamorphosed between June 23 and July 6. Given that the eggs of both populations were laid almost contemporarily, but no overlap for date of metamorphosis was observed, we assumed that all the froglets from ZB metamorphosed at a younger age than did the froglet from MZ. Thus, in our analysis we used population of origin as a factor, considering ZB (the lowland population) as an early metamorphosing population in natural conditions, and MZ (foothill) as a later metamorphosing population. This assumption is confirmed by the observation that we performed during the past years in the field: when tadpoles from both foothill and lowland populations metamorphosed, always lowland tadpoles metamorphosed earlier (Ficetola and De Bernardi, 2005; G.F.F., unpublished).

Interaction terms were tested in preliminary analysis, however they were not included in the final models since they were always non significant (all  $P > 0.1$ ). Bivariate plots were examined to evaluate the presence of non-linearity. We did not observe strong evidence of non-linearity for the relationships between continuous variables within the interval of variation that we examined. Means are reported  $\pm$  standard errors; when descriptive statistics are reported for variables with higher level in the nested design of laboratory study, means and standard error are based on the values on the lower hierarchical level (i.e., the average of a population is based on the average values of five clutches). We did not observe strong violations of model assumptions (normality, homoscedasticity or non-autocorrelation); no cases had an undue influence on the models (all Cook's distances  $< 1$ ) (Bowerman and O'Connell 1990).

## Results

### Laboratory study

In laboratory, the tadpoles from foothill populations grew faster and metamorphosed earlier than did those from the lowland populations (fig. 1; see Ficetola and De Bernardi 2005): the average



**Fig. 1.** Relationship between age at metamorphosis and tibiofibula length in *R. latastei* from five populations. A: data from the 180 individuals developed under standard laboratory conditions; B: average data from the five populations. Error bars equal standard errors. Filled symbols: lowland populations; empty symbols: foothill populations.

**Table 1.** Post-metamorphic traits of froglets grown in nature in early and later metamorphosis populations: descriptive statistics and results of one-way ANOVA. Population ZB: lowland, early metamorphosis in nature; population MZ: foothill, later metamorphosis in nature.

Variable	Mean $\pm$ SE		<i>F</i>	df	<i>P</i>
	ZB	MZ			
Weight (mg)	277.8 $\pm$ 18.3	292.3 $\pm$ 7.50	0.755	1, 37	0.400
Tibiofibula (mm)	6.65 $\pm$ 0.253	7.59 $\pm$ 0.095	15.602	1, 37	<b>0.0003</b>
Max jump (mm)	167 $\pm$ 10.6	245 $\pm$ 10.2	23.466	1, 35	<b>&lt; 0.0001</b>

age at metamorphosis of foothill populations was  $53.4 \pm 0.5$  days; the average age of lowland populations was  $57.2 \pm 1.3$  days. Since some tadpole died prior to metamorphosis, we obtained a total of 180 froglets. At metamorphosis, average tibiofibula length was  $5.41 \pm 0.084$  mm for foothill populations and  $5.69 \pm 0.010$  for lowland populations. Froglets from foothill populations had significantly shorter tibiofibulas ( $F_{1,24.435} = 5.988$ ,  $P = 0.022$ ; Fig. 1); we observed significant variation also among clutches within group ( $F_{21,157} = 1.790$ ,  $P = 0.024$ ).

The ANCOVA showed that, within clutch, the last metamorphosing froglets had the longest tibiofibulas ( $F_{1,156} = 139.866$ ,  $P < 0.0001$ ; Fig. 1); after keeping into account the effect of age at metamorphosis, the differences in tibiofibula length among clutches were also significant ( $F_{21,156} = 3.606$ ,  $P < 0.0001$ ), while differences between the lowland and foothill groups were not significant ( $F_{1,24.809} = 0.049$ ,  $P = 0.826$ ). The strong relationship between age at metamorphosis and tibiofibula length was strong also among clutches, using the average values of each clutch for these variables instead than using data about individual froglets ( $F_{1,21} = 6.302$ ,  $P = 0.02$ ,  $r^2 = 0.231$ ) and among populations, using the average values of each population ( $F_{1,3} = 11.5$ ,  $P = 0.042$ ,  $r^2 = 0.795$ ; Fig. 1b).

The last metamorphosing froglets were the heaviest ( $F_{1,156} = 180.012$ ,  $P < 0.0001$ ); at a given age, the differences in weight between clutches were significant ( $F_{21,156} = 6.637$ ,  $P < 0.0001$ ) but differences between the lowland and foothill groups were not significant ( $F_{1,23.035} = 0.866$ ,  $P = 0.362$ ). Tibiofibula length was strongly positively related to body weight (Pearson's correlation:  $r = 0.823$ ,  $N = 180$ ,  $P < 0.0001$ ); however, keeping equal body weight, the last metamorphosing froglets had proportionally longer tibiofibulas ( $F_{1,155} = 9.053$ ,  $P = 0.003$ ). After keeping into account the effect of age at metamorphosis and body size, we did not find differences in tibiofibula length between foothill and lowland populations ( $F_{1,32.795} = 1.194$ ,  $P = 0.282$ ) or between clutches within population ( $F_{21,155} = 1.279$ ,  $P = 0.197$ ).

### Field study

The froglets from the early metamorphosing population (ZB, lowland) had shorter absolute tibiofibula length and lower jumping performance than the froglets from the later metamorphosing population (MZ, foothill) (Tab. 1, Fig. 2); despite lowland froglets had lower average body weight, differences in body weight were not significant (Tab. 1). Maximum jump was positively related with both weight ( $F_{1,35} = 14.128$ ,  $P = 0.0006$ ,  $r^2 = 0.288$ ) and tibiofibula length ( $F_{1,35} = 44.150$ ,  $P < 0.0001$ ,  $r^2 = 0.588$ ). The froglets from the early metamorphosing population had also (1) shorter size-independent tibiofibula length ( $F_{1,36} = 39.868$ ,  $P < 0.0001$ ;

effect of weight as a covariate:  $F_{1,36} = 109.952$ ,  $P < 0.0001$ ) (Fig. 2A), (2) shorter size-independent maximum jump ( $F_{1,34} = 27.006$ ,  $P < 0.0001$ ; effect of weight as a covariate:  $F_{1,34} = 17.261$ ,  $P = 0.0002$ ) (Fig. 2B), (3) shorter tibiofibula-independent maximum jump ( $F_{1,34} = 5.675$ ,  $P = 0.023$ ; effect of tibiofibula length as a covariate:  $F_{1,34} = 19.711$ ,  $P < 0.0001$ ) (Fig. 2C).

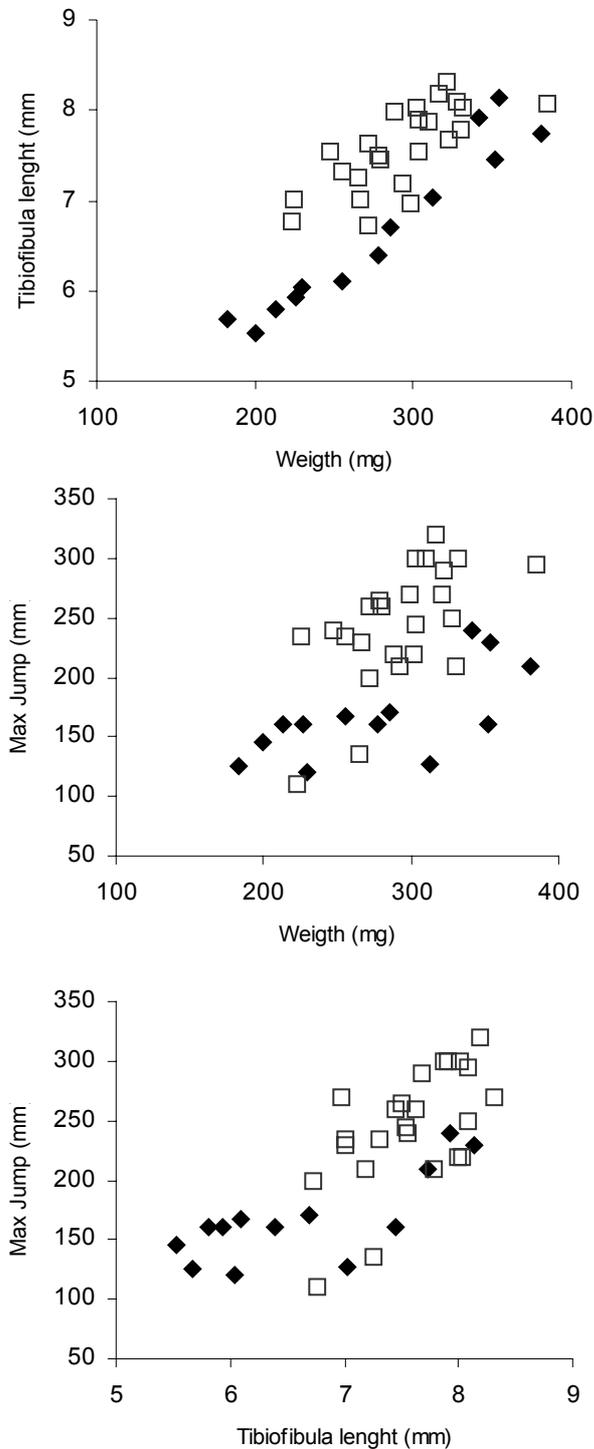


Fig. 2. Relationship between post-metamorphic traits (tibiofibula length, body weight and maximum jump length) in froglets grown in nature. Filled diamonds: population ZB (lowland, early metamorphosis in nature); empty squares: population MZ (foothill, later metamorphosis in nature).

## Discussion

Our results could be summarised as: (1) both absolute and size-adjusted tibiofibula length was larger in the later metamorphosing froglets. This results has been confirmed for froglet grown under laboratory conditions and in the field, despite the differences among froglets in age at metamorphosis had completely different causes in the two environments (that is, in laboratory the later metamorphosing are the froglets with lower intrinsic development rate, while in nature the later metamorphosing are those living in the colder environment). (2) Both absolute and size-adjusted jumping performance were higher in the later metamorphosing froglets. (3) There was a strong, positive relationship between tibiofibula length and jumping performance; anyway, the froglets from the later metamorphosing population hopped longer also keeping equal tibiofibula length.

Altogether, these result support the idea than the later metamorphosing froglets have a strong advantage in terms of locomotor performance if compared to the earlier metamorphosing froglets. That is, the advantages in terms of fitness of earlier metamorphosing tadpoles (i.e., lower risk of predation in the water, lower risk of drying) seems to be balanced by a disadvantage in terms of locomotor performance after the metamorphosis. This trade-off could hinder the spread of the fast development genotype in all the populations (Conover and Shultz, 1995): a tadpole from foothill populations (i.e., a tadpole with fast intrinsic development rate), if transplanted in a lowland wetland where the warm temperature fasten development, could metamorphose very early and thus have short legs and a poor jumping performance, resulting in a scarce ability to catch food and / or escape predators (Zug 1972; Wassersug and Sperry 1977; Heinen and Hammond 1997).

This conclusion is supported by the observation that under laboratory conditions the froglets from foothill populations metamorphosed earlier and had shorter tibiofibula; conversely, in nature the froglets from foothill population metamorphosed later and had longer tibiofibula. Thus, tibiofibula length at metamorphosis, and at least partially jumping performance, seem to be mainly related to the age at metamorphosis, irrespective from the population of origin.

Since we did not measure jumping performance for the froglets reared under laboratory conditions, it could be hypothesized that the froglets from the later metamorphosing population (MZ) jump more than the froglets from the early metamorphosing population for factors others than their age at metamorphosis. However, we showed that both relative and absolute tibiofibula length increase with increasing age at metamorphosis also under laboratory conditions (Fig. 1), and that maximum jump is strongly related to tibiofibula length (Fig. 2). Thus, it is likely that, at least partially, jumping performance is positively related to the time necessary to metamorphosis (see Relyea, 2001; Alvarez and Nicieza 2002).

Several mechanisms may explain the trade-off between larval development rate and post metamorphic traits such as tibiofibula length and jumping performance. In amphibians, tadpoles should reach a minimum size prior that metamorphosis could occur (Wilbur and Collins 1973): if environmental conditions are not limiting for factors such as food availability or density, tadpoles with a delayed metamorphosis can feed for a prolonged period. In turn, these additional resources could be allocated in traits important for post-metamorphic life, such as longer hindlimbs. Moreover, in anurans, thyroid hormones controlling the metamorphosis are the same

controlling hindlimb development. It is possible that a prolonged exposition to these hormones in the last metamorphosing tadpoles caused the observed allometry (Emerson 1986). Finally, in the field study, froglets from the later metamorphosing population had longer maximum jump also after keeping into account the effect of longer legs. This effect could be due to differences other than age at metamorphosis between these two sites, such as differences in food availability. However, Alvarez and Nicieza (2002) showed that froglets reared at lower temperature had more lipid storage: it is possible that differences in energetic reservoirs caused at least partially the differences in jumping performance. A further possible cause of longer jumps in the froglets with slow development is the increase of muscle fibres per muscle unit mass with increasing size or age (Emerson 1978).

It is difficult to find any general pattern relating larval development and post-metamorphic traits in amphibians. A number of studies tried to evaluate if the conditions the larval anurans encounter have consequences on morphology and jumping performance after the metamorphosis. However, only a small subsample of these studies observed a relationship, and there is poor concordance across studies. For example, Relyea (2001) observed that *Rana sylvatica* tadpoles reared with presence of predators have a prolonged larva period and longer legs after the metamorphosis. Conversely, this relationship was not observed for *R. ridibunda* and *Hyla versicolor* tadpoles (Van Buskirk and Saxer 2001; Relyea and Hoverman 2003), despite the use of similar experimental protocols. More generally, some study confirm our results, observing that a prolonged larval period causes longer hindlimbs in metamorphs (Emerson 1986; Relyea 2001; Alvarez and Nicieza 2001), some study did not observe any significant relationship (Blouin and Loeb 1991, Van Buskirk and Saxer 2001; Relyea and Hoverman 2003) and some study observed an apparently opposite pattern, the later metamorphosing froglets having shorter hindlimbs (Blouin and Brown 2000). We can hypothesize that different species of anurans are subjected to different selective pressures, and thus optimisation and covariance between traits can be very different across species and habitats. Moreover, it is possible than tadpoles reared at cold temperatures close to the lower thermal tolerance limit have strong physiological disadvantages as a result of developmental stress (Smith-Gill 1983).

Several studies used the intrinsic larval developmental or growth rate as a surrogate measure of fitness in amphibians, and tried to relate development rate with high genetic diversity, for example to evaluate the presence of inbreeding depression or genetic load (e.g., Rowe *et al.* 1999; Rowe and Beebee 2001; Zeisset and Beebee 2003) or the relationship between maternal investment and fitness (reviewed by Kaplan 1998). Sometimes, these studies dealt with populations tenth or hundred of km apart, distances much greater than those at which countergradient variation has been demonstrated in amphibians (Skelly 2004). Our results show that a fast intrinsic development rate is not equally adaptive in all the environments. Indeed, in some case these studies failed to reveal any relationship between genetic diversity and development rate (e.g., Rowe and Beebee 2001): it is possible that such a relationship should not be expected if different populations have different optimal development rate.

In our study, the cost of fast larval development is evident after the metamorphosis: for species with a complex life history, performance during each stage must be considered to fully understand lifetime fitness (Semlitsch *et al.*, 1999). In complex life cycles, metamorphosis is viewed as a response to selection for developmental independence on different life cycle stages

(the adaptive decoupling hypothesis), because it allow the pre- and post-metamorphic stages to adapt independently to their respective environments, without correlated negative effects (reviewed by Moran 1994). However, our results confirm that larval and adult traits are not entirely evolutionarily independent in amphibians (Watkins 2001), and show that trade-offs could persist also across life history stages, despite the presence of metamorphic events. At least in some case, these trade-offs can explain the persistence of countergradient variation; however, the lack of consistency of this pattern across studies and species suggest that more work is required to evaluate the relationship between metamorphic events and trade-offs.

## 10. CONCLUSION

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This study was an attempt to relate the patterns of distribution and diversity of semi-aquatic herpetofauna to the processes occurring at either local, landscape and regional scale. The seven papers enclosed show that processes occurring at different spatial scales are not independent. Indeed, in a perspective aimed to the conservation of biodiversity, these interactions among processes and spatial scales increase the complexity of management, since a reductionistic approach could not appropriately describe the phenomena occurring in the landscape. That is, the analysis of the effect of a single phenomena on the distribution of semi-aquatic herpetofauna have scarce predictive power; conversely, the interaction among factors explain more and better how each single process occurs (Kiesecker *et al.*, 2002). Here we outline three ensembles of processes that occurred in our study area and that we have showed to be important for the semi-aquatic herpetofauna.

### **Processes driving species distribution**

The chapters 2-4 investigated how the distribution of species and the structure of communities is related to the environmental features measured at both local and landscape scale. These three chapters investigate a wide range of organism: *S. salamandra* is a stream breeding amphibian living on hills; *E. orbicularis* is a pond dwelling reptile living in lowland wetland, and chapter 4 investigated the community of amphibians living in the lowlands of Lombardy. Moreover, also a wide range of habitats has been investigated, including both some of the most natural (the River Po Delta) and some of the most human modified (the province of Milano) areas of River Po lowland.

Interestingly, these three independent studies led to similar conclusions. First, microhabitat features were important for all species. In all these studies, we measured environmental features at very small spatial scale, such as the chemical features of water, the vegetation cover percentage, the presence of basking logs or of submerged woods, the sun exposure. Some of these microhabitat features were important for species distribution: for example, *S. salamandra* breed in the pools with lower phosphate concentration; *E. orbicularis* was observed in wetlands with presence of basking logs, *R. latastei* in wetlands having abundant submerged woods, *H. intermedia* and the newts of genus *Triturus* did not occur in fish occupied wetlands. In some cases these species were observed in wetlands with a suitable microhabitat, but did not occur in wetlands that were only a few meters apart but with unsuitable microhabitat. This selection of microhabitat is likely to be adaptive: for example, organisms can live or breed in wetlands with lower occurrence of predators, with more suitable thermal features, with lower risk of mortality for drowning (see Chapters 2, 3, 4 for references). Many classical studies used wetland features

as a predictors of amphibian distribution (e.g., Beebee, 1985; Pavignano *et al.*, 1990): also differences at very small spatial scale (i.e., within pond heterogeneity) can play a role in the distribution of amphibians (Jacob *et al.* 1998, Tarano 1998).

However, microhabitat selection alone had a poor explanatory power on the distribution of the studied organisms. In our studies, the distribution of *S. salamandra* and *E. orbicularis* was strongly related to landscape features, such as the abundance of natural, wooded landscapes. The importance of terrestrial habitats for semi-aquatic organisms has been frequently overlooked (Gibbons, 2003), however the terrestrial component of ecosystems is vital for herpetofauna (Semlitsch and Bodie, 2003; see also discussion within chapters 2 and 3). Landscape features (i.e., the wetland isolation) play a key role for the distribution of species also for the amphibian communities of Lombardy lowland. Again, processes occurring at landscape scale, such as the extinction-colonisation dynamics of metapopulations (Hanski, 1998), are important to understand species distribution (Marsh and Trenham, 2001). Two studies (Chapters 2 and 3) outlined the importance of wooded landscape for the fire salamander and for the pond turtle: natural wooded landscapes are recognised to be important for many species of semi-aquatic organisms, providing both terrestrial habitat and increasing the connectivity (Laan and Verboom, 1990; Romero and Real, 1996; Hecnar and McCloskey, 1998; Kolozscary and Swihart, 1999; Lemkert and Brassil, 2000; Marchand and Litvaitis, 2004; Porej *et al.*, 2004; Weyrauch and Grubb, 2004).

The most striking result of this thesis for the understanding of distribution pattern of semi-aquatic herpetofauna is the strong interaction occurring between terrestrial and aquatic component of ecosystems: these two components influence each other; for example, the processes occurring in the aquatic component can not be explained without considering the terrestrial one. Frequently, studies about conservation and management of semi aquatic species investigated only the terrestrial or aquatic component alone (reviewed by Marsh and Trenham, 2001; Gibbons, 2003). My study outline that processes occurring at landscape scale can influence microhabitat features important for the distribution of herpetofauna: for example, the presence of a landscape dominated by human activities causes both the unsuitability of terrestrial environment for adult salamanders and increases the pollution in streams (chapter 2). Similarly, the presence of natural woodlands surrounding the wetlands can increases both the suitability of terrestrial habitat for the nesting of turtles, and the suitability of waterbodies for adult turtles (chapter 3). Moreover, we have showed that the interdependence between terrestrial and aquatic habitat can have a synergistic effect on the survival of populations: landscape alteration can make the terrestrial habitat unsuitable; in turn, the modified terrestrial habitat can make less suitable the aquatic environment. Thus, alterations occurring in the terrestrial environment can have a synergistic effect with those occurring in wetlands, increasing the risk of extinction of populations (chapter 2). These considerations have important implications for the management of semi-aquatic organisms. The management of terrestrial habitat should be prioritised, since it can influence the aquatic one. However, the management of terrestrial environment can be more expensive: if natural vegetation would be left in large portions of terrestrial environment, these areas would not be exploitable by human activities. The complex balance between economic development and conservation of ecosystems should be thus kept into account.

### **Isolation and survival of populations**

Isolation is becoming a major factor driving the extinction of populations. We have discussed the increasing occurrence of isolation in human exploited landscapes: natural patches become small and isolated, and isolated populations face of the risk of loss of fitness and extinction for a multiplicity of factors (see chapters 4,7). Isolation is a process occurring at landscape scale, caused by the presence of barriers, by the increased distance among habitat patches and more generally by the loss of connectivity of the landscape (Bennett, 1999). Clearly, species that have low mobility can be more susceptible to isolation: amphibians have reduced dispersal capabilities (see Marsh and Trenham, 2001), thus they can be a useful model to test the consequences of isolation on organisms with reduced mobility. The multiple consequences of isolation on populations can interact among them, thus increasing the risk of extinction: the isolated populations face off both the risk associated to stochasticity (i.e., demographic fluctuations, natural catastrophes or both: Gill, 1978; Laan and Verboom, 1990; Sjørgen, 1990) and the risks associated to the loss of genetic diversity (Hedrick, 2001). The synergism among these factors could increase the risk of extinction of populations: for example, a genetically depleted populations can be more severely affected by environmental alterations than a population with higher genetic diversity (Reed *et al.*, 2002). Thus, a population suffering inbreeding depression can survive under optimal environmental conditions, but become extinct in presence of environmental stochasticities that would only marginally affect outbreed populations. In this studies, I observed that most of amphibian populations surviving in the human dominated areas are not isolated (chapter 4), and that the few isolated populations suffer strong inbreeding depression (chapter 7). This pattern is consistent with the theoretical expectations, and allow to hypothesize a synergism between stochasticities and inbreeding. Moreover, the isolated populations face off a great risk of extinction in the long term, thus management actions are needed. However, the presence of intraspecific diversity among populations suggest care in the selection of the most appropriate management tools (see below).

Clearly, re-colonisation of isolated patches where populations are extinct is impossible. Thus, despite stochastic extinctions occurs naturally in subpopulations within metapopulations, the isolation can cause a reduction of species range at regional scale also in absence of loss of habitat (Hanski and Gilpin, 1997; Bennet, 1999).

### **Local adaptation and the maintenance of intraspecific biodiversity**

At regional scale, this study showed that populations are not homogeneous, and that they can adapt in response of environmental differences among sites (i.e., thermal differences). Biologically significant differences has been observed also despite environmental differences were not so strong: the differences in altitude were  $\sim 100$  m, and the average thermal differences 1-2°C (chapters 8-9). These small environmental and geographical differences would suggest a substantial homogeneity among populations: conversely, my study revealed that foothill and lowland populations are ecologically distinct: they constitute evolutionary significant units (see Crandall *et al.*, 2000) (chapter 8). Which are the forces allowing the maintenance of biodiversity at regional scale? Actually, most of these populations are isolated (see chapter 7), thus the gene

flow is prevented. However, we can hypothesize that prior to the land use expansion that occurred in the last centuries, gene flow were possible among neighbouring populations. Gene flow among populations living in different habitats can reduce the occurrence of local adaptation, since non adapted genes can enter in the populations at an high rate (King and Lawson, 1995). Thus, some mechanism is expected to occurs to prevent the loss of local adaptations: the trade off among traits can be one of these mechanisms. For example, in a lowland environment, foothill individuals would have very fast larval development (a trait believed to correlate well with larval survival), but also very short legs after the metamorphosis and reduced jumping capability (chapter 9). Thus, the spread of these animals in the lowland populations is at least partially prevented, since they would suffer strong fitness disadvantages.

The adaptation to the different optima experienced by populations is a force that has caused differentiation among populations and the increase of adaptive genetic diversity: the different populations have a great conservation value for the maintenance of evolutionary potential of the species (see discussion within chapter 8). Thus, conservation plans should avoid genetic homogenisation: as previously suggested, the management of landscape and the increase of effective population size should be prioritised. Again, the landscape management would be more expensive than translocations, however, it would allow the long term survival of populations. Translocations that are coupled with landscape management would not avoid the risk of extinction.

### **Ecology, genetic diversity and contemporary evolution: open questions**

This study shed light on multiple aspects of ecology and conservation of semi-aquatic herpetofauna; however, several questions are still open, and would need further investigations.

We observed the effects of landscape composition on the distribution of several semi-aquatic species (chapters 2-3), and the effects of landscape configuration (i.e., patch isolation) on the structure of communities. It would be interesting to evaluate the effect of landscape composition on the structure of semi-aquatic communities, to evaluate how wide are the relationships between terrestrial environment and semi-aquatic organisms. Moreover, if among patches colonisation rate is low, species distribution could be also affected by the structure that the landscape had in the past: for example, it is possible that the presence of woodlands is not enough to allow the persistence of *R. latastei* populations. If the recolonisation of patches is hindered by isolation, the patches where populations persist are not only those with the most suitable environment, but also those where a suitable environment has been present with temporal continuity. Further studies are required to explore the importance of temporal continuity of environment for the distribution of species with reduced mobility.

Moreover, we observed reduced fitness in isolated populations, and we assumed this loss of fitness to be caused by inbreeding depression. However, we did not measure the genetic diversity of these populations, thus we do not have genetic data supporting this hypothesis. Indeed, it is possible that the use of neutral markers, such as microsatellite DNA, to reveal the differences in genetic diversity among populations, would not reveal reduced genetic diversity in isolated populations. *Rana latastei* populations living in the western side of the range have a very low genetic diversity: *R. latastei* is among the less genetically diverse amphibians species

investigated (Garner and Tomio, 2001): it is possible that the relationship between genetic diversity measured at neutral markers and fitness of individual would not be related in this species, since a strong genetic depletion occurred earlier, in the post glacial colonisation of this species (Garner *et al.*, 2004a). The causal linkages between genetic diversity estimated at neutral markers and inbreeding depression are not clearly understood (Balloux *et al.*, 2004). The study of a species that lost genetic diversity for natural causes thousand of years ago, and now is facing anthropogenic isolation, could shed light in the effects of extreme isolation.

Finally, it has been recently showed that local adaptation can be very fast in frogs (Skelly and Freidenburg, 2000). Thus, for a species with fast generation time, local adaptation is possible in historical times. It would be very interesting to investigate if this species can adapt to an environment that is changing because of the human alteration, and how fast these modifications are possible. *Rana latastei* would be an excellent model to explore these hypothesis, and to evaluate the interactions between contemporary evolution and the survival of threatened species.

## **Conclusion**

This thesis has revealed the importance of processes occurring at regional and landscape scale for the understanding of distribution and fitness of semi-aquatic herpetofauna at local scale. The complexity of relationships among spatial scales can better explain the processes influencing wild populations, and it should not be overlooked in the management of threatened species-



## ACKNOWLEDGMENTS

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This work would not be possible without the help of many peoples, who helped during the three years of research.

First, I thank all students who helped during field and laboratory work: Valentina Garavaglia, Filippo Gualla, Ilaria Mazzoleni, Alessandro Monti, Laura Nudo and Luca Trotta. Moreover, the chapter 2 would not be possible without the help of Laura Marziali and Prof. Bruno Rossaro; the chapter 3 would not been possible without the help of Emilio Padoa-Schioppa and Luciana Bottoni.

Many more people helped during field and laboratory work, in data analysis, and in reviewing papers. Special thanks to Diego Fontaneto, Roberto Ambrosini, Stefano Scali, Augusto Gentili, Anna Bonardi, Franco Bernini, Gabriele Borsani, Luciano Bani, Marco Baietto, Edoardo Razzetti, Stefano Mazzotti, Roberta Martinelli, Roberta Pennati, Stefano Castiglione, Steven Mullan, Peter Pearman Peter Stockwell, Trent Garner, P.Q. Spinks, L. Sundstrom and several anonymous referees. I also thanks all people who helped my at the Brighton University: Trevor Beebee, Graham Rowe, Eddie Brede, Femmie Kraaijeveld-Smit and Julia Wycherley. Paolo Colombo is the author of the program *Random Matrix Generator*; the open source, freeware statistical software R is available at [www.r-project.org](http://www.r-project.org).

Several parks allowed the access to their territories and to perform field work: Parco Regionale del Delta del Po, Parco Regionale della Valle del Lambro, Parco Regionale di Montevicchia e della Valle del Curone, Parco di Monza, Parco Lombardo della Valle del Ticino, Parco Regionale Adda Sud, Parco Agricolo Sud Milano. The Parco Agricolo Sud Milano partially founded the study of *E. orbicularis* populations. I also thanks several landowners, the municipality of Alfonsine and the Corpo Forestale Dello Stato for allowing to perform this study within several restricted access areas. The *Societas Herpetologica Italica (S.H.I.)* – sezione Lombardia provided inestimable information about the distribution of *R. latastei*; The Italian Ministero dell’Ambiente e della Tutela del Territorio provided the authorisation to collect *R. latastei* eggs and tadpoles (DPN/2D/2004/17391).

This thesis project won in 2003 the F. Barbieri Award promoted by the *S.H.I.*, for a PhD project with herpetological subject.

Finally, a special tank to prof. Fiorenza De Bernardi, for her constant attentions, advices and encouragements, and to Prof. Nicola Saino, Prof. Mario Cotta Ramusino and Prof. Renato Massa. Their help has been indispensable in all phases of this work.



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## RIASSUNTO:

### **DISTRIBUZIONE E DIVERSITÀ DELL'ERPETOFAUNA SEMIACQUATICA IN PIANURA PADANA: DALL'ANALISI DEL MICROHABITAT A UNA PROSPETTIVA REGIONALE**

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Anfibi e rettili semiacquatici sono vertebrati fortemente minacciati a scala globale. Le alterazioni ambientali sono tra le principali cause del loro declino: prima di pianificare interventi gestionali per la loro salvaguardia, è necessario capire come le modificazioni ambientali determinino la distribuzione delle specie, la diversità intraspecifica e la fitness delle popolazioni. In particolare, recenti studi hanno sottolineato come la coazione di più fattori possa spiegare meglio le dinamiche del declino rispetto ai singoli fattori. Questo studio ha indagato gli effetti della struttura ambientale a diverse scale spaziali (microhabitat, paesaggio e scala regionale), e l'interazione tra processi che avvengono a queste scale spaziali sulla distribuzione di anfibi e rettili in pianura padana.

Nella prima sezione della tesi (capitoli 2-4) è stato valutato il ruolo relativo di caratteristiche delle zone umide e del paesaggio ad esse circostante nel determinare la distribuzione e l'abbondanza di diverse specie di anfibi e rettili. Sono stati studiati tre sistemi indipendenti: la distribuzione della salamandra pezzata (*Salamandra salamandra*) nei ruscelli delle colline della Lombardia Occidentale (Cap. 2); la distribuzione della testuggine palustre europea (*Emys orbicularis*) nel Parco Regionale del Delta del Po (Emilia Romagna) (Cap. 3), e la struttura delle comunità di anfibi nell'area circostante la città di Milano (Cap. 4). I tre sistemi hanno fornito risultati coerenti tra loro: le caratteristiche delle zone umide (microhabitat) sono risultate estremamente importanti per la distribuzione di tutte le specie indagate, suggerendo che ogni specie selezioni i siti riproduttivi o in cui vive sulla base delle caratteristiche ambientali. È però risultato evidente che le caratteristiche della zona umida non siano sufficienti a spiegare la distribuzione delle specie. Le caratteristiche dell'ambiente terrestre circostante la zona umida, infatti, hanno in molti sistemi un ruolo preponderante per determinare l'idoneità della zona umida per le specie semiacquatiche. Ovvero, una zona umida con caratteristiche ambientali potenzialmente idonee per una specie può risultare non occupata se l'ambiente circostante ad essa non è idoneo. In particolare, è risultata fondamentale sia per *S. salamandra* che per *E. orbicularis* la presenza di boschi in prossimità di ambienti acquatici. Inoltre, le caratteristiche ambientali delle zone umide spesso non sono indipendenti da quelle dell'ambiente circostante. Ovvero, l'ambiente terrestre può interagire con le caratteristiche delle zone umide, incrementandone o riducendone l'idoneità. Per esempio, i ruscelli circondati da elevata copertura boschiva hanno presentato minore concentrazione di fosfati, verosimilmente perché si trovano in paesaggi meno alterati dall'uomo. Pertanto, l'ambiente terrestre deve essere considerato non solo per la sua importanza per alcuni stadi vitali, ma anche per la sua potenziale influenza sugli ambienti acquatici. Spesso l'importanza dell'ambiente terrestre per l'erpetofauna è stata trascurata: è invece prioritario considerare l'ambiente terrestre nella pianificazione territoriale per la salvaguardia di queste specie.

Nei capitoli 4 e 7 è stata valutata l'importanza dell'isolamento per la distribuzione delle specie di anfibi e per la fitness delle popolazioni. In accordo con i risultati predetti dalla teoria delle metapopolazioni, l'isolamento ha mostrato avere effetti negativi sulla probabilità di occupazione di una tessera di habitat. Ovvero, a pari caratteristiche ambientali, le tessere più isolate hanno presentato una minore probabilità di essere occupate dalle specie di anfibi. Ciò può essere spiegato dal fatto che eventi di estinzione in popolazioni isolate non possono essere compensati da successive ricolonizzazioni. Poiché estinzioni locali avvengono frequentemente all'interno delle metapopolazioni, le popolazioni più isolate hanno un'elevata probabilità di estinguersi anche in assenza di alterazioni ambientali (Cap. 4). Inoltre, le popolazioni isolate vanno incontro a pesanti rischi di incrocio tra consanguinei e deriva genetica. Ciò si può tradurre in perdita di fitness delle popolazioni (depressione da inbreeding). Nel capitolo 7 è stato misurato il tasso di sopravvivenza larvale di popolazioni di rana di Lataste (*Rana latastei*) a diverso tasso di isolamento. In condizioni di laboratorio, le popolazioni isolate hanno presentato un tasso di sopravvivenza pari a un terzo di quello delle popolazioni non isolate. È pertanto verosimile che fenomeni di depressione da inbreeding siano in corso in queste popolazioni, minacciandone la vitalità e la sopravvivenza a lungo termine.

Nel capitolo 5 si è valutato se, a scala regionale, la rana agile (*Rana dalmatina*) possa interferire durante la riproduzione con *R. latastei*, causandone quindi una riduzione nel tasso di fertilità e quindi se possa avere il potenziale di influenzarne la distribuzione. L'ipotesi che interazioni comportamentali durante il periodo riproduttivo tra specie che non danno luogo a accoppiamenti interspecifici possa determinarne la distribuzione è stata raramente testata in natura. Studi di laboratorio hanno dimostrato come in condizioni controllate la presenza di maschi di *R. dalmatina* riduca la fertilità delle femmine di *R. latastei*: ho pertanto valutato se in condizioni naturali le popolazioni di *R. latastei* sintopiche con *R. dalmatina* presentano ridotta fertilità. Non è stata osservata nessuna relazione tra presenza o abbondanza di *R. dalmatina* e fertilità delle uova di *R. latastei*, pertanto è possibile che in condizioni naturali l'interazione tra le due specie sia meno intensa di quanto avvenga in laboratorio. È quindi improbabile che l'interazione tra queste due specie ne determini la distribuzione spaziale a scala regionale.

Infine, nell'ultima sezione della tesi si è valutata la variazione intraspecifica a scala regionale di *R. latastei* per alcuni caratteri considerati adattativi. I dati presentano un quadro estremamente complesso. Come già detto, alcune popolazioni, isolate e nelle colline della Brianza (Lombardia occidentale), hanno una ridotta sopravvivenza agli stadi larvali, verosimilmente per depressione da inbreeding (Cap. 7). Ciò suggerirebbe interventi gestionali per incrementare la diversità genetica in queste popolazioni e quindi ridurre gli effetti della depressione da inbreeding. Due interventi sarebbero ipotizzabili: traslocazioni di individui da popolazioni a maggiore diversità genetica, o interventi di gestione ambientale. Allevando girini provenienti da popolazioni differenti in condizioni di laboratorio, è stato però osservato come i girini provenienti dalle popolazioni della Brianza abbiano una velocità di sviluppo intrinseca più elevato (Cap. 8). Ovvero, se allevati in condizioni identiche, gli individui della Brianza raggiungono la metamorfosi più rapidamente rispetto agli individui provenienti dalla bassa pianura (fiumi Adda e Ticino). Ciò è spiegabile come variazione adattativa (variazione contro gradiente) in risposta a un gradiente termico presente in natura: in natura, le popolazioni della Brianza vivono in un ambiente più freddo rispetto a quelle della bassa pianura. Pertanto, in natura la bassa temperatura ambientale tende a rallentarne lo

sviluppo: la più elevata velocità intrinseca delle popolazioni che vivono nell'ambiente più freddo (Brianza) serve verosimilmente a contrastare gli effetti del gradiente termico sul fenotipo. Pertanto, le popolazioni della Brianza e di bassa pianura sono adattate a condizioni climatiche diverse, e devono essere considerate unità evolutive indipendenti: interventi di traslocazioni sono da evitare, poiché l'omogeneizzazione può causare perdita di diversità genetica interspecifica e potenziale adattativo della specie. Infine, si sono valutati i meccanismi che permettono il persistere della variazione contro gradiente. Le rane a metamorfosi più precoce hanno presentato lunghezza relativa delle zampe minore, e capacità di salto minore, rispetto a rane a metamorfosi precoce. Un velocità di sviluppo larvale rapida può avere dei vantaggi adattativi prima della metamorfosi, ma questi vantaggi possono essere pagati dopo la metamorfosi con una minore performance locomotoria. Pertanto, la variazione intraspecifica sembra mantenuta da bilanci costi-benefici tra caratteri adattativi (Cap. 9). È interessante notare come queste variazioni probabilmente adattative siano state osservate a ridotta scala geografica (massima distanza tra popolazioni: 70 km).

Riassumendo, questa tesi rivela l'importanza dei meccanismi a scala regionale e di paesaggio per la fitness e la distribuzione delle popolazioni anche a scala locale. La complessità delle relazioni tra scale spaziali può influenzare pesantemente le popolazioni di anfibi e rettili semiacquatici, e deve essere presa in considerazione nei progetti di conservazione delle specie minacciate.