

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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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 developmental rate between foothill and lowland *R. latastei* populations, to evaluate if they are evolutionarily 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 hypothesise that counter-gradient selection promoted fast growing phenotypes in a cold environment, where low temperatures slow down larval development. Foothill populations, despite being only a small geographical distance away from lowland populations, seem to be adapted to a colder climate and represent an evolutionarily significant unit. Different populations should, therefore, be managed independently, avoiding translocation. We suggest that evolutionary divergence between populations should be verified prior to planning relocation programmes, to prevent the risk of genetic homogenisation.

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 (for reviews, see Griffith *et al.*, 1989; Fischer & 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 the animals is termed translocation (for definitions, see IUCN, 1995).

In supplementation actions, individuals can be moved in areas where habitat deterioration or hunting has caused a decrease in population size. Supplementation could reduce the vulnerability of recipient populations to environmental and demographic stochasticity, allowing faster population growth and the recolonisation of newly suitable habitat (Lubow, 1996). Moreover, supplementation has been proposed as a tool for managing populations that are facing the problems of inbreeding

depression (Hedrick, 2001). Such small, isolated populations can be threatened by the 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 & 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 in the genetic differences between populations (Moritz, 2002). Intraspecific genetic diversity is recognised as a central conservation problem (Huges, Daily & Ehrlich, 1997; Sinclair, 2001) and the potential for a species' evolutionary success is maximised 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 'evolutionarily significant units', suggesting that they should be independently managed to avoid genetic homogenisation (Ryder, 1986; McKay & Latta, 2002; but see also Moritz, 2002). Evaluating whether different populations are evolutionary independent is, therefore, an important question to resolve prior to supplementation.

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Nevertheless, experimental tests of adaptive divergence between populations are scarce in endangered species (Hedrick, 2001).

Among vertebrates, amphibians are considered by some authors to be potential candidates for relocation programmes (Marsh & Trenham, 2001; Semlitsch, 2002; but see Seigel & 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 on the 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 urbanisation and now *R. latastei* survives only in a limited number of frequently small and isolated woodlands. Moreover, the isolation of suitable patches has increased the probability of extinction of these populations (Ficetola & 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 (Gentili *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, Angelone & Pearman, 2003; Garner, Pearman & Angelone, 2004). 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). 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* in 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 geographical distance between the populations is small (< 60 km); hence the lowland populations could be good sources of individuals for translocation to increase the 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 making 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 to be evolutionarily 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 & Shultz, 1995) and are important traits affecting the 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

Five localities in Northern Italy where *R. latastei* populations live were sampled (Fig. 1). These populations have different geographical settings: three are in the hills of Brianza (AL, CU and MZ) at altitudes of 320, 300 and 175 m above sea level (a.s.l.), respectively; while the remaining two populations (TC and ZB) are found in the 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 (Grossembacher, 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 all are protected in Natural Parks. During early March 2003, we gently removed from each population a small fraction (58 ± 5 eggs) from each of five spawns laid during the night before the sampling day. The eggs were transferred into 200 ml plastic containers and returned 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, Bernini & Barbieri, 2000). At the MZ site an anthropogenic pond drying killed all tadpoles prior to the metamorphosis.

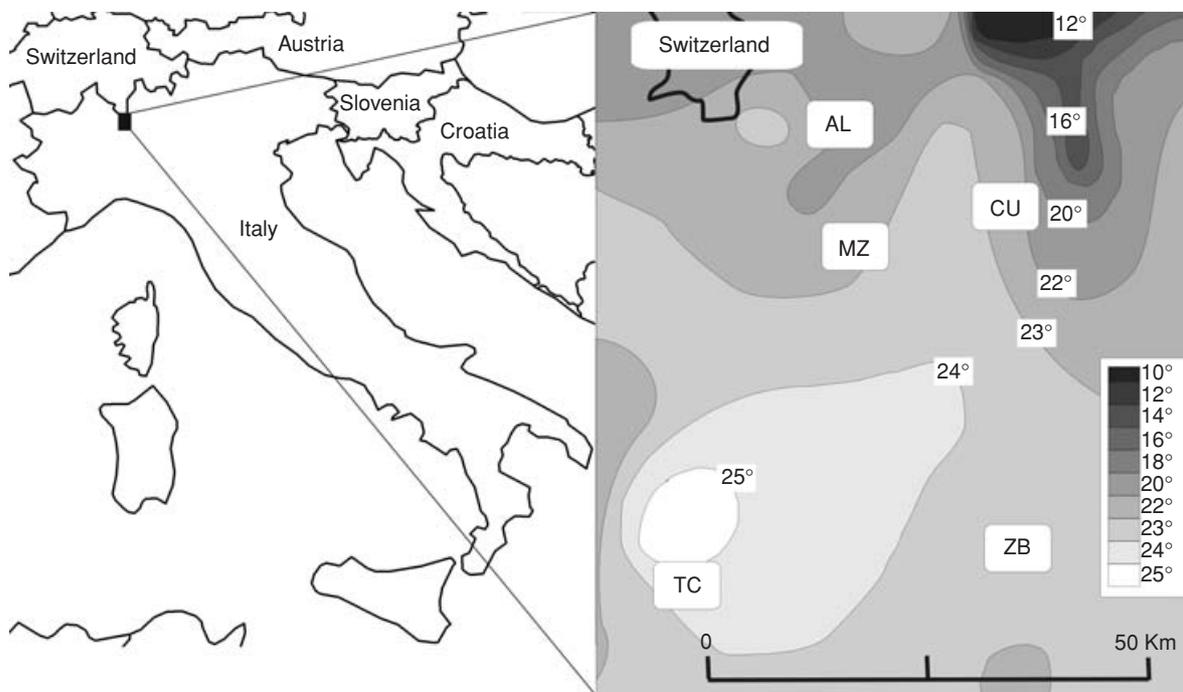


Fig. 1. Location of sampled populations (Lombardy Region, Northern Italy) and average daily temperature ($^{\circ}\text{C}$) in July. Temperature map was redrawn from Belloni & Annovazzi (1999). TC and ZB are lowland sites, AL, CU and MZ are foothill sites.

Larval performance measures in the laboratory

Spawn samples were reared 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, five clutches per population, five 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 the 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 at 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 metamorphosis, (defined as the almost complete tail resorption (Gosner's stage 45)), was recorded and metamorphosis 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 hatching was taken as a measure of tadpole growth in terms of weight gain rate, since soon after this the fastest developing tadpoles began metamorphic climax and lost weight (Fig. 2). We considered that the age at metamorphosis was a measure of developmental rate.

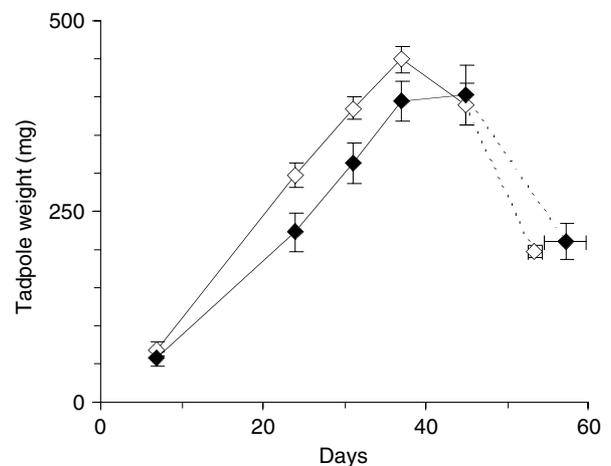


Fig. 2. Growth curves and weight at metamorphosis of tadpoles reared in a common environment. Open diamonds, tadpoles from foothill populations; filled diamonds, tadpoles from lowland populations. Error bars = 2 standard errors; horizontal error bars = 2 standard errors of age at metamorphosis. Weight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

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 a covariate in our analysis.

We used mixed model analysis of variance to evaluate the effects of geographical 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 a covariate to check for maternal effects (see Parichy & Kaplan, 1992; Zeisset & Beebee, 2003 for a similar approach), since tadpole hatching size is strongly correlated with egg size (Kaplan, 1998; Laugen, Laurila & Merila, 2003a). 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 ± 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 17 May. During the same day, the most developed tadpoles we caught in the hill site AL were at developmental stage 33 (toe development of hind limbs). We caught the first metamorph in the hill site AL on 18 June. 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 1 month longer to complete the metamorphosis than did ZB (lowland) tadpoles.

Laboratory study

None of the embryos from two spawn samples of the population CU survived until stage 25, hypothetically due to 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 the foothill populations grew faster and metamorphosed earlier than those from the lowland populations (Fig. 2). Average weight 37 days after hatching was 449 ± 9 mg for foothill tadpoles and 395 ± 13 mg for lowland tadpoles (Fig. 3(a)). The first tadpoles metamorphosed 48 days after 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 ± 0.5 days after hatching while lowland tadpoles metamorphosed 57.2 ± 1.3 days after hatching: development rate was therefore 7.1% faster in the foothill populations (Figs 2, 3(b)). Weight 37 days after hatching

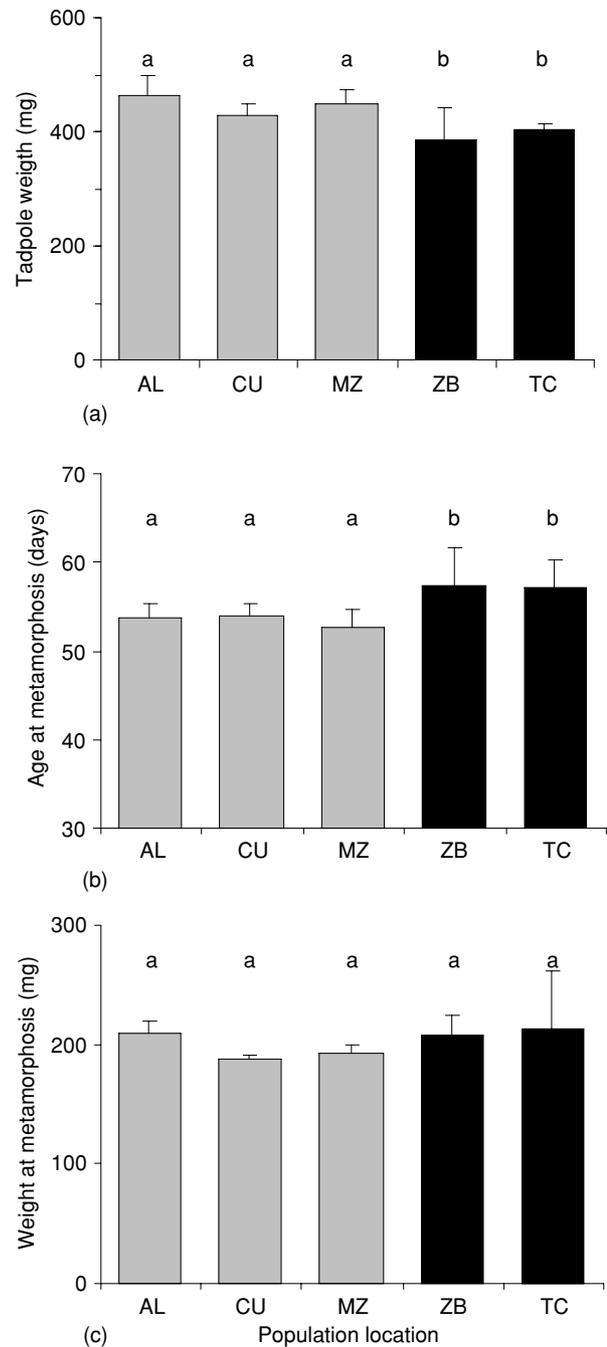


Fig. 3. Larval performance of *R. latastei* tadpoles from five populations. Grey bars, foothill populations; black bars, lowland populations. Error bars = 2 standard errors. (a) weight of tadpoles 37 days after hatching. (b) age at metamorphosis. (c) weight at metamorphosis. ^{a,b}Indicate significant differences for the larval performance measure considered: all populations labelled 'a' are significantly different from all populations labelled 'b'; populations sharing the same letter are not significantly different from one another.

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 ($r = 0.148$, $n = 23$,

Table 1. Effects of various factors on different *R. latastei* tadpole life history parameters

| Factors | F | d.f. | P |
|--------------------------------|--------|-------|-------------------|
| A. Dependent: W _{37D} | | | |
| GEOG | 20.152 | 1,4.7 | 0.007 |
| POP | 0.508 | 3,16 | 0.682 |
| W_START | 0.453 | 1,16 | 0.511 |
| DENSITY | 4.730 | 1,16 | 0.045 |
| B. Dependent: AGE_METAM | | | |
| GEOG | 9.838 | 1,6.1 | 0.020 |
| POP | 0.294 | 3,16 | 0.829 |
| W_START | 0.702 | 1,16 | 0.415 |
| DENSITY | 0.016 | 1,16 | 0.902 |
| C. 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 | < 0.001 |

GEOG, geographical position; POP, population; W_START, starting weight; DENSITY, final density in experimental containers; W_{37D}, weight 37 days after hatching; AGE_METAM, age at metamorphosis; W_METAM, weight at metamorphosis.

$P = 0.50$). 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 geographical positions on larval performance, but differences were not present between populations within groups (Table 1). Tadpole starting weight did not have a significant effect on larval performances, suggesting a lack of egg-size related maternal effect. Tadpoles from foothill populations grew faster and metamorphosed earlier than tadpoles from lowland populations (Table 1, Figs 3(a), (b)). However, geographical position did not seem to influence the weight at metamorphosis (Table 1, Fig. 3(c)). 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 (Table 1). Since we did not observe a significant variability within group for any measure of larval fitness, we repeated the analysis pooling the data from populations with the same geographical position. The results were the same as those 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 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 ($F_{1,19} = 56.476$, $P < 0.001$).

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.*, 2003b). 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 effects (c.f. Laugen *et al.*, 2003b).

Many studies have demonstrated that growth and development rates strongly influence the fitness of amphibians. High growth and development rates enable tadpoles to metamorphose quickly to escape death in drying ephemeral ponds, to escape aquatic predators, or to maximise size at metamorphosis, therefore these traits should be subject to strong natural selection (Wilbur & Collins, 1973; Travis, Keen & Julianna, 1985; Banks & Beebee, 1988; Newman, 1988a,b). 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 affects development and growth rate in amphibians: tadpoles living in a colder environment grow and develop slowly. A few studies have outlined that when compared in a common environment, larvae from populations living in colder areas showed the genetic capacity to complete metamorphosis faster (Berven, Gill & Smith-Gill, 1979; Berven, 1982a; Loman, 2002a). When geographical variation in genotypes is in opposition to environmental influence, a trait displays a countergradient variation (Conover & 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 *R. latastei* live in a slightly colder climate, with average spring and summer temperature being 1–2 °C lower than that for lowland populations (Belloni & 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 a relatively few sites, tadpoles were reared at only one temperature and the populations cannot 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 temperatures more similar to those of their wetland of origin (Skelly & 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 & Collins, 1973; Laurila, Karttunen & Merila, 2002; Loman, 2002*b*). However, none of the wetlands of our study was temporary, therefore this latter hypothesis seems to be unlikely. The desiccation of 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 between sites can not be ruled out, such as differences in predator abundance (Lardner, 1998), differences in food availability or other unrecognised differences between habitats.

Local adaptation: implications for 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 a source of individuals in supplementation projects aimed at increasing the size and/or genetic diversity of the foothill populations. Indeed, translocation could have negative effects for the conservation of *R. latastei*. First, 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 & 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 not be adapted to the warmer climate, suffering a reduced ability to cope with higher temperatures (Skelly & Freidenburg, 2000). Furthermore, it is possible that populations differ for other life-history traits not investigated in this study, such as size and age at first reproduction (Berven, 1982*b*) and, therefore, the home-site advantage could be even stronger. Finally, mixing different populations could

result in the reduced fitness of hybrids, a phenomenon called outbreeding depression (Storfer, 1999; Montalvo & Ellstrand, 2000; Edmands & 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 should allow an increase in the population size, however, the results of these actions will require a long time. During the winter of 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. Gentili, pers. comm.). Obviously, an increase in genetic diversity will require a longer time than 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 a 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 times, resulting in significant adaptive differences being present between populations that are close or living in similar habitats. Many studies have used variation at neutral genetic markers to evaluate differences between populations prior to planning translocations 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 & Ellstrand, 2000; Hedrick, 2001; Pearman, 2001; McKay & 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.

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