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Invasiveness of an introduced species: the role of hybridization and ecological constraints

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Abstract Introduced species are confronted with new environments to which they need to adapt. However, the ecological success of an introduced species is generally difficult to predict, especially when hybridizations may be involved in the invasion success. In western Europe, the lake frog *Pelophylax ridibundus* appears to be particularly successful. A reason for this species' success might be the presence of the invader's genetic material prior to the introduction in the form of a hybrid between *P. ridibundus* and a second indigenous water frog species. These hybrids reproduce by hybridogenesis, only transmitting the *ridibundus* genome to gametes and backcrossing with the indigenous species (i.e. *P. lessonae*). This reproductive system allows the hybrid to be independent

from *P. ridibundus*, and allows the *ridibundus* genome to be more widely spread than the species itself. Matings among hybrids produce newly formed *P. ridibundus* offspring (N), if the genomes are compatible. Therefore, we hypothesize that hybridogenesis increases the invasiveness of *P. ridibundus* (1) by enhancing propagule pressure through N individuals, and/or (2) by increasing adaptation of invaders to the native water frogs' habitat through hybrid-derived *ridibundus* genomes that are locally adapted. We find support for the first hypothesis because a notable fraction of N tadpoles is viable. However, in our semi-natural experiments they did not outperform *ridibundus* tadpoles in the native water frogs' habitat, nor did they differ physiologically. This does not support the second hypothesis and highlights ecological constraints on the invasion. However, we cannot rule out that these constraints may fall with ongoing selection, making a replacement of indigenous species highly probable in the future.

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Ecophysiological tolerance · Larval performance

Introduction

Research on biological invasions focuses on two main topics: the potential of species to become invasive (i.e.

invasiveness), and the susceptibility of native communities and habitats to invasion (i.e. invasibility) (Alpert et al. 2000; Kolar and Lodge 2001; Sakai et al. 2001). So far, this research agenda was unable to clearly define characters for species invasiveness or community invasibility (Facon et al. 2006). Although some successful invaders may be “pre-adapted” to exploit their new environment, most invaders evolve in response to the new selective regime of the invaded habitat (Ellstrand and Schierenbeck 2000; Lee 2002; Facon et al. 2006; Suarez and Tsutsui 2008). This may have hampered the predictability of biological invasions. A general eco-evolutionary framework may improve our understanding of biological invasions. Two general patterns have emerged from several studies: invasions tend to be facilitated by high propagule pressure (Lockwood et al. 2005) and by hybridization with closely related native species (Ellstrand and Schierenbeck 2000). High propagule pressure can be achieved either through a single introduction of a large number of individuals or by frequent introductions (Lockwood et al. 2005, 2007; Kelly et al. 2006; Roman and Darling 2007; Barney and Whitlow 2008). Hybridization between an introduced and a native species, or intraspecific hybridization, may allow for genetic exchange, leading to novel genotypes that may be fitter than parental genotypes in the natural environment, thereby promoting accelerated rates of evolution and range expansion (Rhymer and Simberloff 1996; Ellstrand and Schierenbeck 2000; Facon et al. 2005; Mallet 2005; Schmeller et al. 2005; Culley and Hardiman 2009; Schierenbeck and Ellstrand 2009).

Here, we report on a biological invasion in which both of these effects are relevant, yet in a highly unusual manner. The lake frog, *Pelophylax ridibundus* Pallas 1771 (formerly *Rana ridibunda* until Frost et al. 2006; genotype RR), is native to eastern and north-eastern Europe and parts of Asia (Günther 1990), but it is successfully invading central and western Europe following human translocations (Pagano et al. 2001, 2003; Vorburger and Reyer 2003; Zeisset and Beebee 2003; Schmeller et al. 2007; Holsbeek et al. 2008). In contrast to other invasive species, the genetic material of *P. ridibundus* was already present in the invaded areas. That was possible because *P. ridibundus* produced viable bisexual hybrids with other water frogs, such as *P. lessonae* Camerano 1882 (*R. lessonae*, genotype

LL) and *P. perezi* (Berger 1983). These hybrids, *P. esculentus* Linnaeus 1758 (*Rana esculenta*, genotype RL) and *P. grafi* Crochet, Dubois, Ohler & Tunner 1995 (*Rana grafi*, genotype RP) are assumed to have immigrated after the last glaciation (Wurm age) from areas of sympatry between the parental species. *Pelophylax esculentus* is assumed to have immigrated to France from eastern Europe (Vorburger 2001a), while the immigration history of *P. grafi* remains unclear (but see Arano et al. 1994). Except for some pure hybrid populations where triploid individuals exist and reproduce (Rybacki and Berger 2001; Plötner 2005; Holsbeek et al. 2008; Christiansen and Reyer 2009; Arioli et al. 2010), *P. esculentus* generally occurs in mixed populations with only one of its parental species over large parts of Europe (Uzzell and Berger 1975). In the *Pelophylax* species group, an unusual, hemiclinal mode of reproduction termed hybridogenesis occurs (Schultz 1969). Considering the L-E system, in *P. esculentus*, the *lessonae* genome is usually discarded from the germ line prior to meiosis, which results in the production of functional haploid gametes containing the clonally transmitted *ridibundus* genome (R). Hybrids persist in that system by backcrossing with *P. lessonae* (e.g. Uzzell and Berger 1975; Graf and Polls Pelaz 1989). Such matings combine a sexual *lessonae* with a clonal *ridibundus* genome to form the next generation of *P. esculentus*. The equilibrium in the L-E system was recently thrown off due to introductions of water frogs from the *P. ridibundus* group originating from several countries (mainly Egypt, Turkey and Balkan region to central Europe). This resulted in a high genetic heterogeneity of the introduced frogs because substantial differences exist among taxa within the “*P. ridibundus*” group (Holsbeek et al. 2008; Plénet et al. 2009; Akin et al. 2010; Plötner et al. 2010). Especially in France, the expansion range has increased since the middle of the twentieth century because of repeated introductions of live water frogs (Pagano et al. 2001; Schmeller et al. 2005; but also see Plötner 2005). From an ecological point of view, introduced *P. ridibundus* populations differ from the native water frogs in that they inhabit mainly fluvial and peri-fluvial habitats characterized by renewed water with high dissolved oxygen levels, matching the poor performance of this species under hypoxic conditions (Tunner and Nopp 1979; Pagano et al. 1997; Plénet et al. 1998, 2000a, 2005). In contrast,

the diploid hybridogen *P. esculentus* is constrained to coexist with *P. lessonae* in the L-E system, which preferentially occurs in habitats characterized by high dissolved organic carbon and by variable oxygen availability with severe hypoxic episodes (Plénet et al. 2005).

Many consequences of translocations of *P. ridibundus* in the western European water frog complex (L-E system) exist (Schmeller 2004; Holsbeek and Jooris 2010). Most importantly from a genetic aspect, *P. ridibundus* can also be formed by homotypic matings between two diploid *P. esculentus* individuals (Vorburger 2001c). Such newly formed *P. ridibundus* progeny are often assumed to be unviable (97% mortality) due to homozygosity of deleterious mutations that have accumulated in the clonally transmitted R genomes (Berger and Uzzell 1977; Binkert et al. 1982; Graf and Polls Pelaz 1989; Vorburger 2001a; Guex et al. 2002; Christiansen et al. 2005). However, there is evidence that these newly formed *P. ridibundus* can occasionally survive and even reach maturity, largely depending on the genetic difference between the two haploid R-genomes (e.g. Hotz et al. 1985; Berger et al. 1988; Hotz et al. 1992; Vorburger 2001c; Guex et al. 2002; Schmeller et al. 2005). The invasion of introduced *P. ridibundus* into native L-E systems creates novel opportunities for primary hybridizations between *P. ridibundus* and *P. lessonae*, which may boost the diversity of *ridibundus* genomes among hybrids (Pagano et al. 2008). Consequently, the viability of newly formed *P. ridibundus* offspring is expected to increase, leading to an amplification of the *P. ridibundus* propagule pressure and thus to enhanced invasiveness (Guex et al. 2002; Vorburger and Reyer 2003, Schmeller et al. 2005). In addition to just increasing propagule pressure, newly formed *P. ridibundus* may also play a role in increasing local adaptation of the invaders. This may occur if there is occasional recombination between *ridibundus* and *lessonae* genomes in hybrids. This generally does not occur in native L-E systems (but see Mezhzherin et al. 2004; Schmeller 2004), yet it has been shown to be common in primary hybrids between introduced *P. ridibundus* and native *P. lessonae* or *P. perezi* (Uzzell et al. 1977; Pagano and Schmeller 1999; Guex et al. 2002; Vorburger and Reyer 2003;

Schmeller 2004; Schmeller et al. 2005; Som and Reyer 2006; Vorburger et al. 2009). Another relevant genetic mechanism is the introgression of *lessonae* mtDNA into *P. ridibundus* (Spolsky and Uzzell 1984; Hotz et al. 1992; Plötner et al. 2008). Because of these gene exchanges, newly formed *P. ridibundus* may be more likely to cope with ecological conditions usually favouring the indigenous water frogs, *P. esculentus* and *P. lessonae*.

Despite their potential importance, the consequences of newly formed individuals on the invasiveness of *P. ridibundus* are little explored so far (but see Vorburger and Reyer 2003). Therefore, we experimentally compared the larval performance of newly formed *P. ridibundus* with that of introduced *P. ridibundus* and native *P. esculentus* in situ across ponds differing in relevant ecological characteristics (i.e. *P. lessonae*- and *P. ridibundus*-typical habitats), as well as in the laboratory under standardized conditions (only comparison between the two *ridibundus* types). We also compared ecophysiological characteristics (i.e. tolerance to hypoxia) between introduced and newly formed *P. ridibundus*. We argue that if newly formed *P. ridibundus* perform similarly to introduced *P. ridibundus* and *P. esculentus* independent of environmental conditions, they could be considered as a novel *P. ridibundus* source increasing propagule pressure. If they outperform introduced *P. ridibundus* under *lessonae*-typical conditions or hypoxia, an additional role in improving the local adaptation of the *P. ridibundus* population would be supported.

Materials and methods

Study animals and taxon identification

We used tadpoles of three types: introduced *P. ridibundus* (hereafter noted R), newly formed *P. ridibundus* (N, produced by matings between hybridogenetic *P. esculentus*) and the hybridogen *P. esculentus* (E, produced by matings between *P. esculentus* and *P. lessonae*). The R clutches came from invasive *P. ridibundus* populations located near Lyon where only *P. ridibundus* adults were found (Pagano et al. 2001). In this study, we used the name *P. ridibundus sensu lato* ('marsh frogs') for all water frogs of the *P. ridibundus* superspecies. The real

phylogenetic status of *P. ridibundus* remains complex, as it comprises several cryptic allo- and parapatric species with very similar morphology (e.g. Plötner et al. 2001). The N (R-like clutches) and E clutches were collected in ponds located near Lyon where only *P. lessonae* and *P. esculentus* adults were found (L-E system; Plénet and Joly, unpublished data).

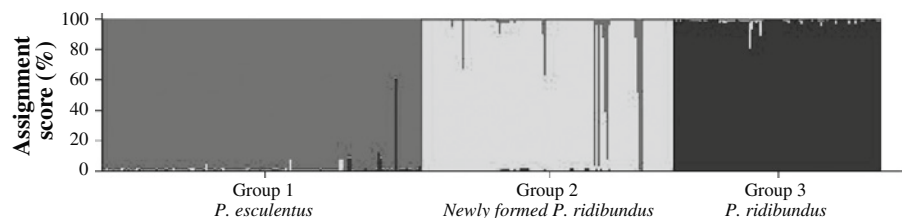
Prior to the experiment, we analysed two tadpoles per clutch to distinguish between R, L and E clutches using allozyme electrophoresis with the locus LDH-B. LDH-B is a locus with no allelic overlap between *P. ridibundus*, *P. lessonae*, and *P. perezi* (see Uzzell and Berger 1975; Pagano et al. 1997) and with little impact from recombination events between the *ridibundus* and *lessonae* or *perezi* genome (Schmeller 2004 and references cited therein; Schmeller et al. 2005). This method confirmed the R status of clutches sampled in pure introduced *P. ridibundus* populations. In L-E populations, allozyme electrophoresis allowed us to distinguish R-like, L and E clutches. Because only *P. lessonae* and *P. esculentus* adults were found in these studied populations (L-E system; Plénet and Joly, unpublished data), we considered the R-like clutches as the newly formed *P. ridibundus* resulting from hybrid *P. esculentus* matings (N status). Despite the absence of *P. ridibundus* in these specific ponds, the R hemigenomes possessed by the hybrids may well be influenced by the invasion of *P. ridibundus* in the area. They are likely to represent a mixture of genetic material present in the hybridogens prior to invasion and new genetic material that arrived with the introduction of *P. ridibundus* (see Introduction). At the end of the experiments, to confirm the N status, toe clips from all froglets successfully metamorphosed and preserved in 96% ethanol were analysed with three polymorphic microsatellite loci: R1Ca1b5 (Garner et al. 2000), Res16 and Res17 (Zeisset et al. 2000), with sufficient polymorphism and absence of null alleles in *P. ridibundus*. We followed extraction protocols from Lalouette et al (2010), and PCR

protocols from Garner et al. (2000) and Zeisset et al. (2000). The genotyping was done on a MegaBACE™ automated sequencer, and analysed using Genetic Profiler™. From the populations used in this study, 148 E individuals, 117 N individuals, and 96 R individuals were genotyped. We used the program STRUCTURE to validate the E, N and R groups, using 3 assumed populations (E, N, R), population admixture, allele frequencies independent, burn-in length of 10,000, and run length of 100,000. All runs were replicated eight times, with K ranging from 2 to 7. K = 3 groups maximised posterior probabilities. As evident in Fig. 1, very few individuals were assigned to other groups, and were most probably from mixed E and N broods (no wrong assignment for a priori R individuals).

In situ experiment

In our in situ experiment, we compared the larval performance of R, N and E tadpoles in both R-typical and L-E-typical natural habitats using permeable enclosures (60 × 50 × 40 cm polyethylene tanks; mesh = 1 mm; for more details see Plénet et al. 2005). The two ponds used in this study were located 20 km apart in the same region (i.e. Rhône-Alpes region, South-Eastern France) with homogeneous climatic conditions. The R-typical habitat was a perfluvial pond in the Rhone floodplain (45°48' 1.3"N, 4°53'14"E), where water is continuously renewed by underground flow and characterised by low dissolved organic carbon and high dissolved oxygen levels (Pagano et al. 2001; Plénet and Joly, unpublished data). In contrast, the L-E-typical habitat was a pond without any fluvial influence (45°56' 43.2"N, 4°55'8.5"E), characterised by a silted bottom, high dissolved organic carbon levels and variable oxygen concentrations close to normoxia (Plénet and Joly, unpublished data). It is exclusively inhabited by L-E mixed populations. The tadpoles used in the

Fig. 1 STRUCTURE summary plot of estimates of a posteriori probability of assignment to the original group. Each bar represents one individual



experiment were not collected at these experimental sites to avoid confounding effects due to local adaptation.

For every tadpole type (R, N, and E), we collected 10 freshly laid clutches from two source populations (5 clutches per population) on the same day. The clutches were maintained individually in nursery tanks in the laboratory under standardized conditions. When tadpoles reached the same developmental stage (Gosner stage 25, Gosner 1960), we mixed four tadpoles originating from five clutches from each source population and each tadpole type, to form six different groups of 20 individuals. These six groups were replicated three times per pond. The 36 groups of 20 tadpoles were placed in enclosures in the R-typical and the L-E-typical ponds (18 groups per pond). Groups of tadpoles were assigned randomly to enclosures within ponds. The enclosures were positioned in the ponds so that they received equal amounts of sunlight and experienced similar physical conditions (distance to the shore and to vegetation). They were floating so that water depth was maintained at 20 cm (approximately 54 L volume) throughout the experiment. The enclosures were stocked on 3 June 2004 and checked every 2 days thereafter until 15th September 2004. When tadpoles had both forelegs (i.e. Gosner stage 42–43), they were removed from enclosures and reared individually in the laboratory until the end of metamorphosis, i.e. until tail resorption was complete (stage 46). The experiment was ended when only few non-metamorphosed tadpoles remained in the enclosures. We determined several measures of larval performance: survival (the proportion of individuals surviving until the end of the experiment, including tadpoles that had not yet metamorphosed), metamorphosis success (the proportion of survivors that successfully completed metamorphosis), larval period (time from the start of the experiment until completion of metamorphosis), and two estimates of froglet body size: wet body mass and snout-to-vent length.

Laboratory experiment

In our laboratory experiment, we compared the larval performance of N and R tadpoles under benign laboratory conditions. As in the in situ experiment, N and R tadpoles came from several clutches from the same two source populations (N individuals from two

to five clutches, R tadpoles from four to five clutches per population). We reared 100 N and 100 R tadpoles individually (50 tadpoles per source population per tadpole type). We randomly selected between 10 and 25 tadpoles per clutch. In early June 2006, when tadpoles had reached stage 25, they were placed individually in boxes (12 cm diameter) filled with 1 L of tap water. These boxes were randomly distributed in a room that was maintained at 23°C (\pm 1°C) and with a 14:10 h light–dark cycle. We fed tadpoles with boiled lettuce ad libitum every day and changed the water in each box every 2 days. When tadpoles reached stage 42–43, they were reared individually in a terrarium until the end of metamorphosis (stage 46). The experiment was terminated on 15th September 2006 when only few tadpoles had not yet entered metamorphosis. We took measurements of larval performance twice during their development. At mid development (41 days), we measured total length (to the nearest 0.1 mm using a binocular equipped with an ocular micrometer), wet body mass (to the nearest 0.1 mg; tadpoles dried on filter paper), and developmental stage according to Gosner (1960). At metamorphosis, we measured the same traits as in the in situ experiment.

Physiological performance experiment

Tadpoles of introduced *P. ridibundus* are more sensitive to low dissolved oxygen levels than tadpoles of native *P. lessonae* and *P. esculentus* (i.e. higher metabolism, lower LT50 in anoxia and lower metabolic levels; Plénet et al. 2000a). Consequently, *P. ridibundus* occurs mainly in habitats characterised by high oxygen availability and the absence of hypoxic episodes. In order to determine the abilities of N tadpoles to successfully develop in hypoxic habitats, we compared physiological performances of N and R tadpoles. We measured the individual standard metabolic rate (rates of oxygen consumption VO_2), individual lethal time in anoxia and individual metabolite contents of tadpoles (glycogen stored under hypoxic episodes, triglycerids and proteins stored under trophic constraints) using established methods described by Hervant et al. (1995) and Plénet et al. (2000a). Sample sizes varied among assays and were between 10 and 26 individuals per type (N or R) and population. The tadpoles originated from the same clutches as those used in the laboratory

experiment. However, they were reared in groups (mixing several clutches) until they reached developmental stage 37–38 (i.e. beginning of metamorphosis). After the metabolic assays, each tadpole was measured (total length), staged, and weighed (wet body mass).

Statistical analyses

We performed linear mixed model analyses of variance (ANOVAs Type III) for larval period and mass at metamorphosis of tadpoles from the in situ experiment using the nlme library version 3.1–79 (Pinheiro and Bates 2000) in the software R 2.6.0 (R Development Core Team 2007) to compare larval performance between R, N and E tadpoles, accounting for variation among habitats, populations and enclosures. To remove overall size effects on mass, we used the residuals of a log–log regression of mass at metamorphosis on snout-to-vent length. Size-corrected mass is an interesting fitness correlate reflecting individual body condition. Habitat and tadpole type were considered fixed effects. Because only two populations per type were used, population was also treated as a fixed effect, nested within tadpole type (Snijders 2005). Enclosure was considered a random effect. We tested for the effects of habitat, tadpole type, and population, as well as for the habitat \times type interaction and the habitat \times population (type) interaction. The unit of observation for statistical analyses was the mean value of all individual tadpoles within an enclosure. When the condition of homogeneity of variances was not fulfilled, we specified the lme function from the nlme library to account for heteroscedasticity in the within-group errors (Pinheiro and Bates 2000).

Survival and metamorphosis success were analysed with a generalized linear model using the logit link and a binomial error distribution. For this, we used the function glmmPQL (MASS library) in R. The model included the same fixed and random effects and interactions as the ANOVAs for larval period and mass at metamorphosis.

For our laboratory experiments, the statistical analyses were similar to those previously described. We performed mixed model ANOVAs (Type III) to compare larval stage, larval period, tadpole mass at mid development and at metamorphosis between N and R tadpoles. Tadpole mass at mid development

and at metamorphosis were again size-corrected, and we allowed for heterogeneity of variance in our models. Analyses to test differences in survival and metamorphosis success between the two types of *P. ridibundus* were conducted using a generalized linear mixed model. Tadpole type and population were considered fixed effects, clutch was a random effect nested within population.

Finally, the physiological performances of tadpole types and populations (nested within type) were analysed on the basis of the three physiological variables (VO_2 , lethal time in anoxia, and metabolite content) by nested ANOVAs.

Results

In our in situ experiment, survival of R, N and E tadpoles was similar ($F_{2, 22} = 1.897$, $P = 0.174$), and survival was similar between populations ($F_{6, 22} = 1.609$, $P = 0.192$). However, survival of R, N and E tadpoles differed strongly between habitats ($F_{1, 22} = 74.571$, $P < 0.001$). In the habitat typical of native L-E populations, survival was high for all tadpoles (>80%; Table 1) whereas it was low for all tadpoles in the R-typical habitat (<50%; Table 1).

Metamorphosis success was 100% for all types of tadpoles in the L-E-typical habitat and was significantly higher than in the R-typical habitat (Table 1). Interestingly, a higher proportion of E tadpoles metamorphosed successfully in enclosures of the R-typical habitat than either R tadpoles ($t = 15.074$, $df = 22$, $P < 0.001$) or N tadpoles ($t = 14.078$, $df = 22$, $P < 0.001$). This resulted in a significant main effect of tadpole type ($F_{2, 22} = 165.644$, $P < 0.001$) as well as a significant type \times habitat interaction ($F_{2, 22} = 3.822$, $P = 0.037$), and a population effect at the threshold of significance ($F_{6, 22} = 2.538$, $P = 0.051$).

Table 1 Survival and metamorphosis success of the three tadpole types in both habitats

Types	Survival (%)		Metamorphosis success (%)	
	R-typical	L-E-typical	R-typical	L-E-typical
R	41 \pm 23.7	92 \pm 9.8	82 \pm 22	100
N	34 \pm 10.8	87 \pm 12	76 \pm 23	100
E	56 \pm 23.5	91 \pm 9.7	91 \pm 6	100

Values are means of enclosures \pm 1 SE

Our tadpole performance analysis showed no difference in size-corrected mass between tadpole types and populations (Table 2; Fig. 2). However, size-corrected mass differed significantly among habitats according to tadpole type (significant interaction, Table 2; Fig. 2). The uncorrected mass of R and N tadpoles reared in the R-typical habitat was three times lower and the snout-vent length about 1.5 times lower than that of tadpoles reared in the L-E-typical habitat. Performance differences between habitats were smaller in E tadpoles.

The effect of the habitat on larval period differed strongly between tadpole types (Table 2; Fig. 2). While for R and E tadpoles, the larval period was almost 2 weeks shorter in the L-E-typical habitat compared to the R-typical habitat, N tadpoles took approximately 1 week longer to develop in the L-E-typical habitat than in the R-typical habitat. This resulted in a significant main effect of tadpole type, as well as a significant type \times habitat interaction (Table 2). In the R-typical habitat, larval period of N was significantly shorter relative to that of E ($t = 18.076$, $df = 22$, $P < 0.001$) and of R ($t = 14.041$, $df = 22$, $P = 0.004$). Conversely, in the L-E-typical habitat, larval period of N was longer relative to that of E ($t = -8.952$, $df = 22$, $P < 0.001$) and of R ($t = -7.326$, $df = 22$, $P =$

Table 2 Mixed-model nested ANOVAs (Type III) for body mass at metamorphosis (using the residuals of a regression performed on logarithm of body mass versus the logarithm of the length interpreted as a measure of size) and larval period (days from the start of the experiment until completion of metamorphosis) of tadpoles reared in the in situ experiment

Source	Num. df	Den. df	F value	P
Body mass				
Intercept	1, 396		0.002	0.959
Habitat	1, 22		0.492	0.490
Type	2, 22		2.448	0.109
Habitat \times Type	2, 22		3.729	0.040
Habitat \times Pop(Type)	6, 22		2.072	0.098
Larval period				
Intercept	1, 396		560.763	<0.001
Habitat	1, 22		11.220	0.003
Type	2, 22		4.122	0.030
Habitat \times Type	2, 22		11.175	<0.001
Habitat \times Pop(Type)	6, 22		1.527	0.216

The random enclosure effect are not shown

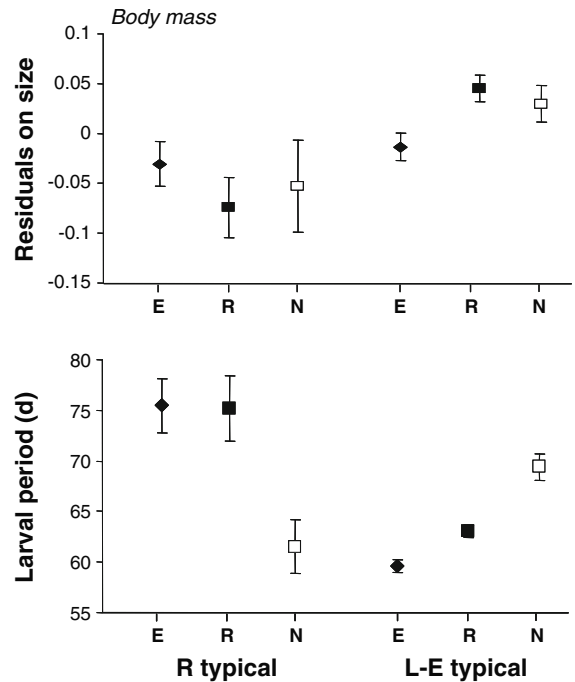


Fig. 2 Body mass corrected on size and larval period of three tadpole types reared in R-typical and L-E-typical habitats. E denotes the hybrid *P. esculentus*, R the introduced *P. ridibundus*, and N the newly formed *P. ridibundus*. Values are means of enclosures and errors bars depict ± 1 SE

0.003). The population effect was not significant (Table 2).

In our laboratory experiment, all tadpoles survived until mid-development (41 days), but fewer N than R tadpoles survived until the onset of metamorphosis ($61 \pm 25\%$ and $98 \pm 3.5\%$ respectively; $F_{1, 12} = 8.424$, $P = 0.013$). The metamorphosis success was high and not significantly different for the two types of *P. ridibundus* tadpoles (N: $86 \pm 38\%$, R: 100% ; $F_{1, 12} = 1.446$, $P = 0.252$).

Despite significant variation between populations within tadpole types, size-corrected mass of N individuals (tadpoles or metamorphs) was slightly (tadpoles) or substantially (metamorphs) larger than that of R individuals (Table 3; Fig. 3). Developmental stage of tadpoles at mid-development and larval period differed strongly between populations within types (Table 3). Larval period also differed among types of tadpoles with a shorter larval period for N than for R individuals (Table 3; Fig. 3). These results suggest that for most of the measured traits (except

Table 3 Mixed-model nested ANOVAS (Type III) for body mass and larval stage of tadpoles at mid-development, body mass and larval period at metamorphosis in the laboratory experiment

Source	Num. df,	Den. df	F value	P
<i>Mid-development</i>				
Body mass				
Intercept	1,	175	19.368	<0.001
Type	1,	12	11.939	0.005
Pop(Type)	2,	12	11.416	0.002
Stage				
Intercept	1,	175	19083.98	<0.001
Type	1,	12	0.759	0.401
Pop(Type)	2,	12	5.940	0.016
<i>Metamorphosis</i>				
Body mass				
Intercept	1,	145	13.327	<0.001
Type	1,	11	13.020	0.004
Pop(Type)	2,	11	1.878	0.199
Larval period				
Intercept	1,	145	1635.72	<0.001
Type	1,	11	5.649	0.037
Pop(Type)	2,	11	15.033	0.001

Body mass was corrected for size as in Table 2. The random clutch effect are not shown

for developmental stage), N tadpoles can outperform R tadpoles.

The physiological parameters, metabolic rate ($F_{1, 100} = 0.697$, $P = 0.406$), lethal time in anoxia ($F_{1, 58} = 1.037$, $P = 0.313$), and metabolite contents (glycogen, protein and triglycerid; $F_{1, 32} = 0.891$, $P = 0.352$; $F_{1, 34} = 1.518$, $P = 0.226$; $F_{1, 62} = 0.300$, $P = 0.586$, respectively) did not differ significantly between N and R tadpoles (Fig. 4). There was, however, significant variation between populations within tadpole types for lethal time in anoxia ($F_{2, 58} = 4.838$, $P = 0.011$) and triglycerid content ($F_{2, 62} = 10.34$, $P \leq 0.001$).

Discussion

We hypothesized that hybridization and hybridogenesis may increase the invasiveness of the introduced water frog *P. ridibundus* in two ways: (1) by increasing propagule pressure through the formation of viable *P. ridibundus* individuals from homotypic

matings among hybrid *P. esculentus*, or (2) by increasing adaptation of the invader to the native water frogs' habitat through providing hybrid-derived *ridibundus* genomes that are locally adapted. While our finding that N tadpoles were viable and comparatively fit supports the first hypothesis, we do not find any support for the second hypothesis. N tadpoles did not differ from R tadpoles in their physiological tolerances, nor did they outperform R tadpoles in the native water frogs' habitat. However, shifts in the relative performance of the tadpole types across different environments support ecological constraints on the invasion of *P. ridibundus*. We discuss each of these three aspects in turn.

Hybridization and propagule pressure

The invasiveness of an introduced species is enhanced by a high propagule pressure, be it through single introductions of a large number of individuals or through frequent introductions (Lockwood et al. 2005; Kelly et al. 2006; Roman and Darling 2007; Barney and Whitlow 2008). Our study provides evidence for another peculiar way to increase propagule pressure that is a consequence of hybridogenesis. Homotypic matings among *P. esculentus* hybrids produce non-hybrid individuals of the introduced species *P. ridibundus*. How important this mechanism is as a source of *P. ridibundus* hinges on the viability and performance of such newly formed *P. ridibundus*. Indeed, their evolutionary fate is arguable. Within populations, *P. esculentus* × *P. esculentus* matings often produce inviable offspring, owing to homozygosity for recessive deleterious mutations (Berger and Uzzell 1977; Binkert et al. 1982; Graf and Polls Pelaz 1989; Vorburger 2001a; Guex et al. 2002; Christiansen et al. 2005). However, several studies found such offspring to be viable and to reach maturity under favorable environmental conditions (all female offspring; Hotz et al. 1985, 1992; Berger et al. 1988; Vorburger 2001c; Guex et al. 2002; Vorburger and Reyer 2003). In these studies, metamorphosed female RR froglets and sexually mature RR females from hybrid × hybrid matings were observed in Swiss populations. Our results concur with these last studies. The newly formed *P. ridibundus* from within-population matings among hybrids survived and metamorphosed at rates largely higher (>70%) than expected based on some rates suggested in the literature (i.e. 3%; Guex et al.

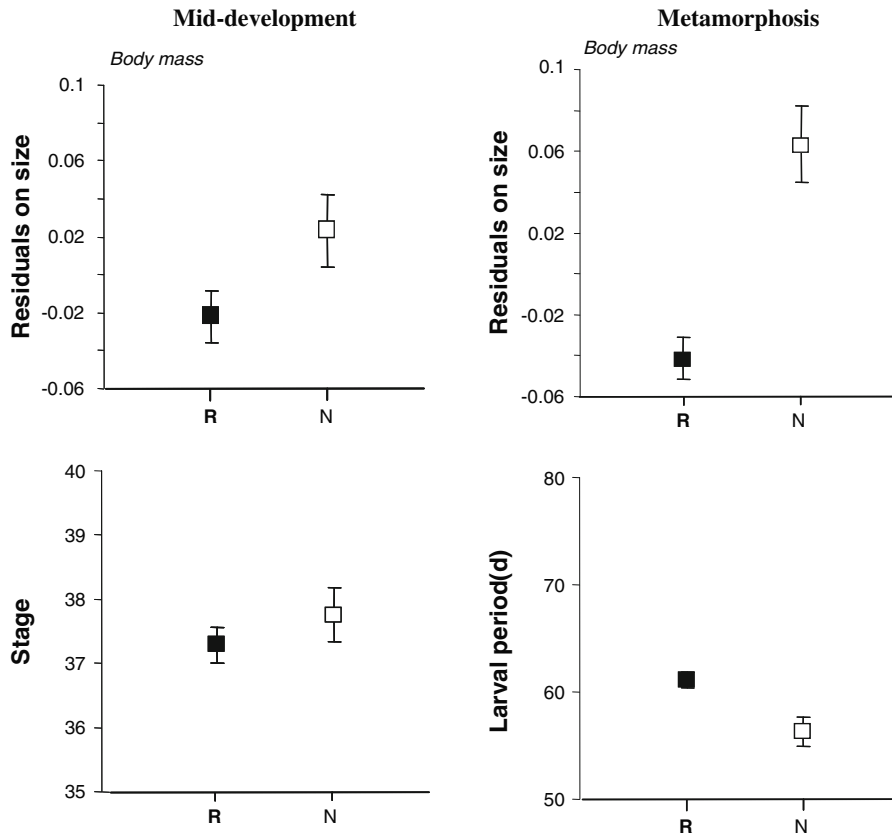


Fig. 3 Body mass corrected on size and larval stage according to Gosner (1960) at mid-development; body mass and larval period at metamorphosis of both *P. ridibundus* types in laboratory conditions. Values are means of clutches and errors bars depict ± 1 SE

2002; but see Schmeller et al. 2005). Moreover, N tadpoles were similar in viability and performance to “normal” *P. ridibundus* and native *P. esculentus* tadpoles under field conditions. Under laboratory conditions, survival of newly formed *P. ridibundus* tadpoles was slightly but significantly lower than that of introduced *P. ridibundus*. On the other hand, the survivors metamorphosed earlier and with a better body condition than tadpoles of introduced *P. ridibundus*. Even if only the tadpole performance until metamorphosis was considered in our study, there is evidence that larval or metamorphic traits do translate into fitness differences in the terrestrial life stage of frogs (i.e. carry over effects; Räsänen et al. 2003). Indeed, in anurans, early metamorphosis and large metamorph size enhance fitness by translating into higher juvenile survival and earlier maturity (Smith 1987; Berven 1990; Altwegg and Reyer 2003), suggesting that newly formed *P. ridibundus* should be quite competitive at postmetamorphic stages.

Another important aspect of the introduction of *P. ridibundus* is that backcrosses between the hybrid and the introduced species as well as matings between newly formed and introduced *P. ridibundus* generate offspring that possess one clonal and one sexual R-genome. The limited evidence available suggests that such offspring are just as fit as sexually produced offspring from R \times R crosses (Vorburger 2001b), and may therefore contribute to the invasive potential of the introduced water frog *P. ridibundus*. However to estimate the full contribution of this process, additional crossings such as R \times E, N \times R or N \times E would be necessary.

Finally, this relatively bright picture of the prospects for *P. ridibundus* produced by homotypic matings among hybrids has to be moderated. The N clutches we used in the experiment came from a larger collection of clutches of which 80% did not hatch (Luquet and Plénet, pers. obs.). It is not impossible that these aborted clutches represented

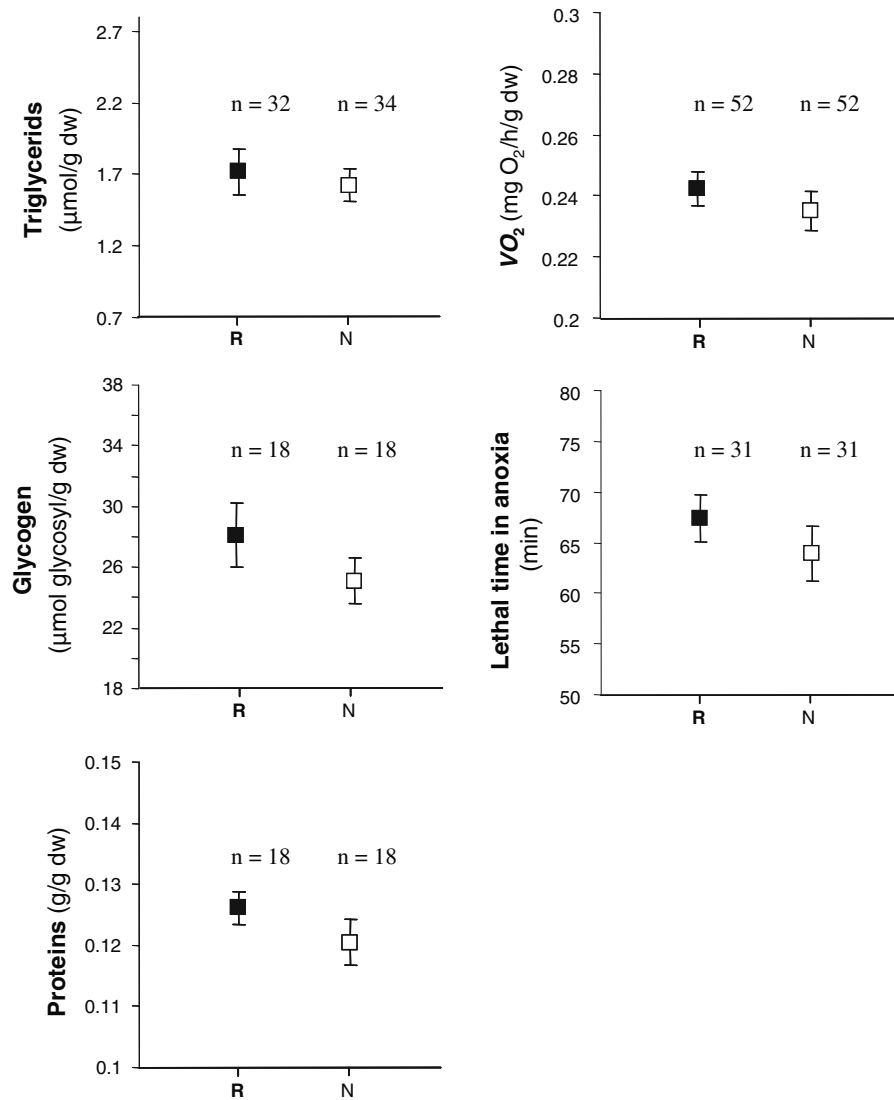


Fig. 4 Metabolic contents (triglycerids, glycogen and protein contents), rates of oxygen consumption (VO_2), and lethal time in anoxia of both *P. ridibundus* types. *n* = numbers of individuals. Values are means and errors bars depict ± 1 SE

unviable hybrid \times hybrid matings. Yet this does not negate the fact that a substantial fraction of such matings produces seemingly fit *P. ridibundus* that can contribute to the propagule pressure of this introduced species. Here, one needs also to keep in mind that water frog females can lay several thousands of eggs.

Hybridization and local adaptation

Hybridization and hybridogenesis may further enhance invasiveness of *P. ridibundus* by improving

the local adaptation of the *P. ridibundus* populations. That is because *ridibundus* genomes present in hybrids are exposed to selection in L-E-typical habitat, and because of the potential gene exchanges between the different taxa of the water frog complex and their effect on offspring fitness and their ecology (Schmeller 2004). Episodic recombination between *ridibundus* and *lessonae* genomes occurs in hybrids (Uzzell et al. 1977; Pagano and Schmeller 1999; Guex et al. 2002; Vorburger and Reyer 2003; Mezhzherin et al. 2004; Schmeller 2004; Schmeller et al. 2005; Som and Reyer 2006; Vorburger et al.

2009). Recombination in hybrids, even if rare, could entail that *ridibundus* genomes present in hybrids are better adapted to the ecological conditions typical for native water frogs than *ridibundus* genomes of introduced populations (Schmeller et al. 2005). Moreover, introgression of *lessonae* mtDNA in hybrid lineages may result from occasional successful matings between hybrid males and LL females. Subsequent mating between *P. esculentus* females with *lessonae* mtDNA and *P. esculentus* (or *P. ridibundus*) males lead to *P. ridibundus* progeny with *lessonae* mtDNA (Spolsky and Uzzell 1984; Hotz et al. 1992; Plötner et al. 2008). That introgressed *lessonae* mtDNA genomes may also provide an adaptive advantage to *P. ridibundus*, and perhaps to the hybrid individuals, is a possibility worth investigating (Plötner et al. 2008).

At our study sites in the Rhone floodplain, adults of introduced *P. ridibundus* inhabit and reproduce in habitats that are connected to freshwater sources (brooks, rivers, freshwater channels) and are characterized by relevant ecological variables such as high oxygen availability or low salinity (Pagano et al. 1997; Plénet et al. 1998, 2005; Plénet and Joly, unpublished data). These sites differ substantially in their ecological characteristics from typical habitats of the native water frogs, which frequently experience severe hypoxia (Pagano et al. 1997; Plénet et al. 2005; Plénet and Joly, unpublished data) matching the higher tolerance of native water frogs to such conditions (Plénet et al. 2000a). Indeed, under reduced oxygen availability, larvae of *P. lessonae* developed better than *P. ridibundus* larvae (Plénet et al. 2000a, b) and the survival time of LL metamorphs was higher than that of RR metamorphs (Tunner and Nopp 1979; Lutschinger 1988). All these results demonstrate that *P. ridibundus* is more sensitive to oxygen deficiency than *P. lessonae*. Consequently, to be successful in *P. lessonae*/*P. esculentus* habitats, tadpoles must be adapted to low-oxygen availability by exhibiting a low metabolism, high LT50 to anoxic conditions and high energetic metabolite content (Plénet et al. 2000a, b). Based on this, we expected N tadpoles to be less sensitive to oxygen deficiency and to exhibit physiological performances that exceed those of R tadpoles (i.e. lower metabolic rate, higher glycogen rate, higher LT50). However, our results provide no evidence for such an effect. Physiological performance and tolerance of

anoxia of newly formed and introduced *P. ridibundus* tadpoles were very similar. Thus, newly formed *P. ridibundus* are expected to be as sensitive to hypoxic episodes as introduced *P. ridibundus*. Similarly, the in situ experiment revealed that N tadpoles did not outperform R tadpoles in the L-E-typical habitat. N tadpoles reached similar body mass at metamorphosis but grew slower than R tadpoles, indicating that they are not better adapted to the ecological conditions in the native water frogs' habitat.

Ecological constraints

Based on the hypothesis of ecological constraints on the invasion of *P. ridibundus*, we expected R tadpoles to perform better in the R-typical habitat but worse in the L-E-typical habitat than the native *P. esculentus* tadpoles. However, this is not what we observed in our in situ experiment. R tadpoles performed just as well as E tadpoles in enclosures in the R-typical habitat, which—based on high mortality and small size of surviving metamorphs—represented the harsher of the two environments for any tadpole type. Clearly, the R-typical habitat was much less productive, which was also evident from the lack of algal growth in these enclosures (Luquet and Plénet, pers. obs.). Considering that stressful environments are thought to amplify differences between genotypes and to exacerbate the negative effects of deleterious mutation accumulation (Kondrashov and Houle 1994; Shabalina et al. 1997; Hedrick and Kalinowski 2000; Szafraniec et al. 2001), it was surprising that in terms of development time, N tadpoles performed best of all three types in the R-typical habitat. Our finding confirms that tadpoles from hybrid × hybrid crosses can be comparably fit. However, an astonishing pattern emerged from the analysis of development time in the more productive L-E-typical habitat. In contrast to E and R tadpoles, which both had a shorter development time in the L-E-typical habitat, N tadpoles needed more time to complete metamorphosis (Fig. 2). We presently lack a satisfying explanation for this interesting habitat × tadpole type interaction.

That the larval performance of *P. ridibundus* was comparable to native species in enclosures of the L-E-typical pond suggests a high invasiveness of this species in such habitats. However, by using just a single L-E-typical pond, we did not cover the whole

range of ecological conditions used by native water frogs. The ecological conditions in our study pond clearly were not at the tolerance limit for *P. ridibundus* tadpoles. Mixed L-E populations are known to occur in habitats that are much more extreme than the one tested here, especially in terms of oxygen availability (i.e. severe hypoxia; Pagano et al. 1997; Plénet et al. 2005; Plénet and Joly, unpublished data). Thus, the ability of *P. ridibundus* to successfully invade all the habitats of autochthonous *P. esculentus* and *P. lessonae* depends on their physiological performances. Previous studies (Tunner and Nopp 1979; Plénet et al. 2000a, b) have concluded that because of a high metabolism, a low LT50 to anoxic conditions and low energetic metabolite contents, the introduced *P. ridibundus* were too sensitive to tolerate the ecological conditions of more extreme L-E habitats and that its tadpoles cannot develop successfully under such conditions. Similar physiological performances between introduced *P. ridibundus* tadpoles and *P. ridibundus* tadpoles resulting from matings among *P. esculentus* may suggest ecological constraints to the invasion of both *P. ridibundus* types at least in some habitat types, as also indicated by the distribution of *P. ridibundus* in the lower Rhone floodplains (Schmeller et al. 2007).

Conclusion

In addition to the spread of *ridibundus*-like frogs in Europe promoted by a high variability and ecological factors, our study suggests that the genetic mechanism of hybridogenesis contributes to the invasion success of the introduced water frog *P. ridibundus*. To consolidate this hypothesis, it would be appropriate to test it using a larger number of ponds per typology and to cover the whole range of the L-E-typical habitat of native frogs in a large scale experimental set up. The hybridogenetic hybrid *P. esculentus* is not just an evolutionary “dead end” for clonal *ridibundus* genomes (Milinski 1994). Hybrids can enhance the invasive potential of *P. ridibundus* because a notable fraction of *P. esculentus* × *P. esculentus* matings within L-E populations produces viable and competitive *P. ridibundus* offspring. Therefore, newly formed *P. ridibundus* may be a significant source of *P. ridibundus* and should be considered in future studies on *P. ridibundus* invasiveness. Our findings

agree with the model developed by Vorburger and Reyer (2003) on the expected dynamics of such a genetically driven species replacement; pure *P. ridibundus* populations are an almost inevitable outcome under ecological conditions suitable for *P. ridibundus*. However, introduced as well as newly formed *P. ridibundus* could be similarly limited to habitats without the occurrence of hypoxic episodes. To confirm these last points, testing and comparing R, N, E and L tadpole performance using a larger number of ponds per typology and increasing the range of L-E-typical habitats would be appropriate. For the conservation of native water frogs, our results are reassuring. Some habitats may function as refuge environments for the autochthonous species. However, we cannot exclude that future genetic exchanges between *ridibundus* and *lessonae* genomes and natural selection processes will eventually allow *P. ridibundus* to invade new habitats.

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