Functional Ecology

British Ecological Society

Functional Ecology 2013, **27**, 459–471 doi: 10.1111/1365-2435.12049

Genotype-temperature interactions on larval performance shape population structure in hybridogenetic water frogs (*Pelophylax esculentus* complex)

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Summary

1. The evolutionary potential and ecological importance of interspecific hybrids continues to be a controversial issue. Traditionally, hybridization – often associated with polyploidy and clonal reproduction – was considered an important mechanism for speciation in plants, but not in animals. More recently, investigations have shifted to the question: Under which genetic and ecological conditions do hybrid taxa and different ploidies arise and succeed, and when and where do they fail? Finding answers to this question is aggravated by the fact that suitable taxa for such studies are often far apart on the phylogenetic tree. Hence, results are influenced by many confounding variables.

2. In this study, we reduce this problem by investigating the fitness within a complex of three closely related water frog taxa consisting of the two sexually reproducing parental species *Pelophylax lessonae* (genotype LL) and *P. ridibundus* (RR) plus their interspecific hybrid *P. esculentus* which comes in three ploidy types (LR, LLR and LRR), as well as with sexual and hemiclonal reproduction. Offspring of all five genotypes were produced by artificially crossing adults sampled from populations in Slovakia, Germany and Switzerland. This created genetic variation. They were then raised at two temperature levels: 18 and 24 °C. This created ecological variation. Larval performance under the two temperature regimes was analysed with respect to three fitness-related parameters: survival rate, days to metamorphosis and weight at tail resorption.

3. Survival rate was significantly higher for offspring of the three hybrid types (LR, LLR and LRR) compared with those of the parental species (LL, RR), at both rearing temperatures. For days to metamorphosis and weight at metamorphosis, we found an interaction between offspring type and temperature. In both cases, performance of hybrid and parental offspring did not differ at 24 °C, but at 18 °C hybrids metamorphosed faster and at a lower weight than parentals.

4. We discuss these results in relation to those from other studies and conclude that under cold conditions hybrids (especially the two triploid types) have higher fitness than both parental species. This genotype × environment interaction could be one reason why all-hybrid populations mainly occur at the cooler northern range of the water frog distribution.

Key-words: all-hybrid populations, clonal reproduction, genetic compatibility, hybridogenesis, larval development, metamorphosis, *Pelophylax esculentus*, ploidy, survival, temperature

Introduction

The evolutionary potential and ecological importance of interspecific hybrids has been a controversial issue for quite some time. While seen as an important process for speciation by botanists (Grant 1981), zoologists traditionally dismissed hybridization as an evolutionary dead end (Mayr 1963). The diverging views originated from the, on average, higher number of successful hybrids in plants than in animals (reviewed by Grant 1981; Arnold 1997; Mallet 2005). However, within both kingdoms, hybridization is very unequally distributed among taxa, and in

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some animals, its rate even exceeds that in plants (Grant & Grant 1992; Ellstrand, Whitkus & Rieseberg 1996; Mallet 2005). Hence, analyses of the evolutionary and ecological role of hybridization should extend the specific comparison between plants and animals to the more general question: What traits and environmental conditions separate taxa with successful hybridization from those where it does not occur in the first place (prezygotic selection) or leads to unfit offspring (postzygotic selection)?

Important features that are often linked to hybridization are clonal reproduction and polyploidy, that is the existence of three or more complete chromosome sets, rather than two. Recent crossing experiments by Choleva *et al.* (2012) with spined loaches (*Cobitis*) suggest that clonality may be 'directly triggered by interspecific hybridization and that polyploidy is a consequence, not a cause, of clonality'. The probability of establishing a successful, that is evolutionary significant, polyploid hybrid lineage is a function of several factors. These include prezygotic ones like the rate at which unreduced gametes are produced and the likelihood that they will fuse. They also include postzygotic ones like the viability and fertility of the resulting offspring and their competitive ability, relative to offspring of the parental species (cf. Vrijenhoek 1989; Soltis & Soltis 1999; Otto & Whitton 2000; Coyne & Orr 2004; Mable 2004). In this study, we focus on the postzygotic factors that determine the success of polyploids, once they have been formed.

Whether polyploid zygotes are produced and develop into viable and fertile polyploid offspring depends on genetic compatibility between the genomes of the two species that hybridize ('balance hypothesis'; Moritz *et al.* 1989). Even when phenotypically viable, allopolyploids (i.e. those arising from hybridization between different species) are often genetically unfit, due to meiotic problems. The fact that approximately two-thirds of allopolyploid animals have abandoned recombination between the parental genomes and reproduce clonally testifies to the importance of avoiding meiotic disturbances (reviewed by Vrijenhoek *et al.* 1989; Beukeboom & Vrijenhoek 1998; Otto & Whitton 2000). Solving the meiotic problem through shifting to clonal reproduction comes at the expense of reduced genetic diversity available for adaptation and the risk of accumulating deleterious alleles through Muller's ratchet (Muller 1964). This is why several authors have considered polyploids and clonal organisms 'evolutionary dead ends', at least as far as individual lineages are concerned (e.g. Vrijenhoek 1989; Maynard Smith 1992).

Even when the genetic problems can be overcome, successful establishment of allopolyploid hybrids may be prevented for ecological reasons. As hybrids are usually intermediate in their characteristics and requirements, they will compete with both parental species that are adapted to and usually superior in different niches along an ecological gradient.

Despite these problems, some clonal and hemiclonal hybrids are ecologically and evolutionary fairly successful (for recent reviews see Arnold 1997; Kearney 2005; Avise 2008; Hörandl 2009; Vrijenhoek & Davis Parker 2009). Ecological explanations for the success of hybrids assume that they can reduce competition by inhabiting different, intermediate or broader niches than their progenitors (Moore 1984; Otto & Whitton 2000; Seehausen 2004). Genetic explanations for the success include (i) occasional incorporation of new nuclear material from the sexual host (Hedges, Bogart & Maxson 1992; Spolsky, Phillips & Uzzell 1992; Schartl *et al.* 1995), (ii) formation of different clonal lineages through repeated primary hybridization (Moritz *et al.* 1989; Ptacek, Gerhardt & Sage 1994; Little & Hebert 1997; Soltis & Soltis 1999; Janko, Kotlik & Rab 2003; Stöck *et al.* 2005), (iii) spontaneous heterosis ('hybrid vigour'; Lippmann & Zamir 2007) and (iv) 'transgressive segregation, that is the production of extreme phenotypes that exceed the combined range of trait values of both parental lines (Rieseberg, Archer & Wayne 1999; Stelkens & Seehausen 2009). Mechanisms (iii) and (iv) are based on the fact that the combination of different parental genomes and/or the addition of extra genomes can lead to increased levels of somatic heterozygosity in hybrids. This may explain why allopolyploids and other hybrids seem to be better adapted than the parental species to invade and establish themselves in novel, perturbed and extreme habitats and, as a result, are found in high proportions at the geographical periphery of species ranges and in harsh environments at high latitudes and altitudes (Otto & Whitton 2000; Mable 2004; Seehausen 2004).

Most of the above conclusions stem from interspecific comparisons, often between taxa that are far apart on the phylogenetic tree. As such comparisons are strongly affected by several confounding variables, comparisons within species or complexes of very closely related organisms are to be preferred for investigating how genetics and ecological competition affect the success of clonally reproducing hybrids with different ploidies, relative to their sexual parental species. However, with a few exceptions (Cullum 1997; Alves, Coelho & Collares-Pereira 2001; Pala & Coelho 2005; Stöck *et al.* 2005, 2010), this has rarely been attempted for vertebrates, because the necessary intraspecific variation is lacking.

THE *PELOPHYLAX* STUDY SYSTEM

An excellent system for such a comparison is provided by the Edible Frog *Pelophylax esculentus* (called *Rana esculenta* until Frost *et al.* 2006). Originally derived from matings between the pool frog *P. lessonae* (phenotype L and genotype LL) and the marsh frog *P. ridibundus* (R, RR), *P. esculentus* (E, LR) combines hybrid origin with hemiclonal reproduction and – in some populations – with polyploidy (LLR, LRR). This allows intraspecific comparisons between hybrids of different ploidies and intracomplex

comparisons between hybrids and their two closely related parental species.

Prior to meiosis, *P. esculentus* eliminates one of the parental genomes, duplicates the remaining genome and transmits it clonally to eggs and sperm cells ('hybridogenesis'; Schultz 1969; Tunner 1974; Uzzell, Hotz & Berger 1980; Graf & Polls Pelaz 1989). As a result of this gametogenesis mechanism, hybrid × hybrid matings lead to larvae of the parental type whose genome is clonally transmitted, that is to RR in case of R genome transmission and to LL in case of L genome transmission. ('hybridolysis', Günther & Plötner 1988; Plötner 2005). However, these parental types of hybrid origin usually do not survive to metamorphosis, because recessive lethal alleles have accumulated on the clonally transmitted genome through the Muller's ratchet mechanism (Berger 1977; Graf & Müller 1979; Uzzell, Hotz & Berger 1980; Vorburger 2001a). Hence, *P. esculentus* is a sexual parasite that must live in sympatry and backcross with the parental species (sexual host) whose genome it eliminates (Schmidt 1993; Joly 2001).

Depending on the specific genetic interactions between the hybrid and the parental species, three major breeding systems can be distinguished: the LE-, RE- and EE-system (Graf & Polls Pelaz 1989; Günther 1990; Plötner 2005). The most widespread and best investigated one is the LE-system, where the hybrids exclude the L genome and breed with *P. lessonae* to re-establish hybridity at each generation (Table 1a). The mirror system to this is the RE-system where hybrids exclude the R genome and backcross with *P. ridibundus* to perpetuate themselves (Table 1b). The all-hybrid EE-system (Table 1c), with no parental sexual host to mate with, seems to defy the rules and mechanisms outlined above. Yet, such populations have been found in several parts of Europe, and they remain stable over many years (Christiansen *et al*. 2010). The key to their success is the existence of and mating between diploid (LR) and triploid hybrids (LLR, LRR). In EE populations, the triploids replace the parental species as sexual hosts by providing the haploid L (LLR) or R gametes (LRR) that in LE and RE populations are produced by LL and RR individuals, respectively.

Table 1. Offspring types (within bold frame) expected from typical gamete types (in italics) and mating combinations that are possible in a) LE-systems, b) RE-systems and c) an EE-systems consisting of diploid LR and two types of triploids, LLR and LRR

a) LE-system	**Males**	LL	LR
Females	***Gametes***	*L*	*R*
LL	*L*	LL	LR
LR	*R*	LR	RR

b) RE-system	**Males**	RR	LR
Females	***Gametes***	*R*	*L*
RR	*R*	RR	LR
(LR)	(*L*)	(LR)	(LL)

c) EE-system	**Males**	LLR	LR	LRR
Females	***Gametes***	*L*	*R*	*R*
LLR	*L*	LL	LR	LR
LR	*LR* *R*	LLR LR	LRR RR	LRR RR
LRR	*R*	LR	RR	RR

Offspring types in grey fields do not occur among adults, although they are initially produced. The brackets in b) indicate that in most RE-systems female hybrids and the resulting gamete and offspring types do not occur.

When genotypes, sex ratios and gamete production patterns are included, several variations of these three basic breeding systems are found in Europe. As a result, the composition of a population (defined by the genotypes of the occurring animals) does not always enable us to deduce the breeding system (defined by genetic interactions) it belongs to. In this study, we therefore speak of population types, rather than breeding systems.

In populations where diploid hybrids occur in sympatry with parental species (in this study represented by an $LE_{2n}R$-population from Slovakia and an LE_{2n}-population from Switzerland), both sexes of all genotypes usually produce haploid gametes. In all-hybrid populations (in this study represented by an $E_{2n}E_{3n}$-population from Germany), there is some variation in gamete types of diploids hybrids, but the most frequent pattern is the one shown in Table 1c (based on Jakob 2007; Christiansen 2009). Considering this pattern of gamete production and all possible mating combinations, we not only expect LR, LLR and LRR hybrids from hybrid × hybrid matings, but also offspring of both parental species, *P. lessonae* (LL) and *P. ridibundus* (RR) (Table 1c). In all-hybrid $E_{2n}E_{3n}$-populations, these do actually occur during larval stages, but no longer exist among adults (Arioli 2007).

In this study, we investigate genetic and ecological factors that might affect the composition of water frog populations via fitness differences between offspring of the two parental species and those of diploid and triploid hybrids. Larvae were produced by artificially crossing adults from different geographical regions, different breeding systems and of different ploidies. This introduced genetic variation. They were raised under two temperatures, which introduced ecological variation. Fitness was measured by three variables that in amphibians are known to represent good correlates: tadpole survival, time to metamorphosis and weight at metamorphosis. Results from the experiment are used to discuss why some water frog populations are mixed, with parental species and hybrids living in sympatry, whereas others consist of hybrids only.

Materials and methods

SOURCE POPULATIONS

The adults used for crossing originated from three European countries (Fig. 1). In Germany, frogs were caught from the village pond of Schönermark (52°54′08″N, 12°19′16″E), near Kyritz; in Switzerland in a pond near Hellberg (47°17′36″N, 8°48′29″E),

Canton of Zurich; and in Slovakia from four ponds, all located within 10 km of Šaštín-Stráže (48°37′55″N, 17°08′40″E). The Kyritz population is an all-hybrid $E_{2n}E_{3n}$-population with diploid LR and triploid LLR and LRR. The Šaštín-Stráže population consists of diploid LR and both parental species, LL and RR ($LE_{2n}R$-population). The Hellberg pond represents an LE_{2n} population where the diploid hybrids occur in sympatry with only one parental species (LL). In all three areas, the mentioned genotypes occur in both sexes, but from the Hellberg pond, only LL females were included in the crossing design.

Frogs were caught at night by hand while dazzling them with a strong flashlight. Sex was determined on the spot based on the presence (males) or absence (females) of thumb pads and vocal sacs. For preliminary genotype determination, we used the shape of the *Callus internus* and produced blood smears on slides for subsequent measurement of erythrocyte length and width. This allows identification of ploidy because triploids have larger cells than diploids (Polls Pelaz & Graf 1988; Jakob 2007), but it does not allow unambiguous distinction between individuals of the same ploidy, that is between LL, RR and LR or between LLR and LRR. Therefore, all frogs were toe clipped for later genotype identification through microsatellite analysis. For transport to Zurich, selected frogs were individually marked with transponders (ID-162, AEG), separated by sex and assumed genotype, stored in cloth bags filled with rubber sponges and showered daily with fresh water. All frogs survived the journey.

MICROSATELLITE ANALYSIS

Precise genotype identification of both the parental frogs and the offspring resulting from the crosses was achieved through microsatellite analysis using a piece of the tailfin (tadpoles) and a fingertip (adults, metamorphs), respectively, as the source material. DNA extraction and purification were performed using a Biosprint 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) in combination with the Biosprint 96 workstation following the supplier's protocol. The purified DNA was subjected to PCR runs with four primer mixes involving a total of 18 microsatellites primer pairs (Table 2). Details on PCR protocols are given by Christiansen (2009) and Christiansen & Reyer (2009, 2011). PCR products were run for fragment length analysis on an ABI 3730 Avant capillary sequencer with internal size standard (GeneScan-500 LIZ), and the alleles were scored with the Genemapper software v3.7 (Applied Biosystems, Zug, Switzerland). Loci Res20, RlCA1a27 and RlCA18 were species-specific for *P. lessonae*, and Res22, Rrid169A and Re2CAGA3 were species-specific for *P. ridibundus*. The other 12 loci amplified in both *lessonae* and *ridibundus* genomes (Christiansen 2005, 2009; Arioli, Jakob & Reyer 2010). Moreover, loci CA1b6, RlCA1b5, Ga1a19redesigned and Res16 showed a dosage effect which was used to detect triploidy by comparing the relative height of the peaks (Christiansen 2005). Knowing the genotypes of the parents and their offspring, we could infer the genotype and ploidy of the gametes they originated from. This also allowed us to check for possible aneuploidy of the offspring, which did not occur.

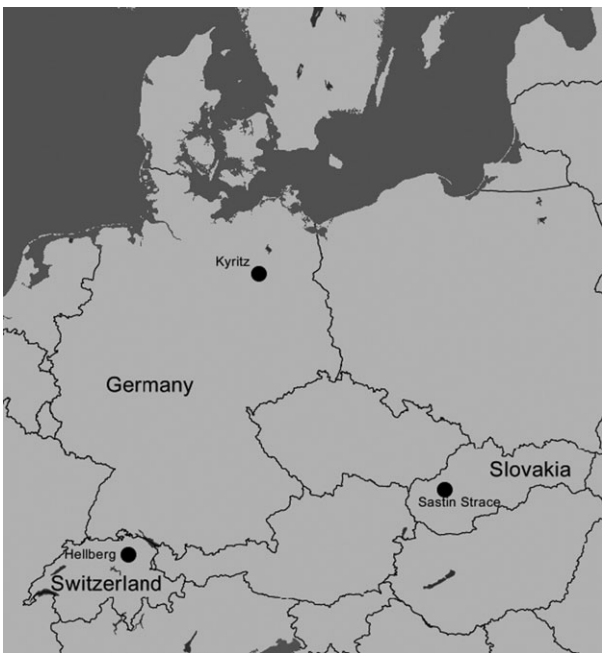

Fig. 1. Locations of sampled populations near Kyritz (Germany), Hellberg (Switzerland) and Šaštín-Stráže (Slovakia) with the following airline distances between them: Kyritz – Hellberg 680 km, Kyritz – Šaštín-Stráže 585 km, Hellberg – Šaštín-Stráže 640 km.

CROSSING PROCEDURE

Crosses were performed through artificial fertilization, following the protocol of Berger, Rybacki & Hotz (1994) with the following modifications. To stimulate ovulation, females were injected with LHRH fish hormone (Bachem H-7525; 2 mg in 100 ml Holtfreter's solution). After about 24 hours, when eggs were ready for being stripped off, males were euthanized in a buffered (pH 7) MS-222 solution (A-5040; Sigma, St. Gallen, Switzerland) at 1 mg L^{-1}. Their testes were removed and a piece crushed in a Petri dish with aged tap water. Eggs were stripped into this sperm suspension, where they remained for about 2–3 min. After this period, the suspension was rinsed into a new Petri dish to which eggs of another female were added. This allowed using the same sperm solution to fertilize eggs from different females. After fertilization, eggs were covered with aged tap water and checked for fertilization success. This can be easily identified because fertilized eggs rotate their black animal hemisphere to the top within 30–60 min.

The next day, all eggs were transferred to containers with 1 litre of water with a water–air interface of 600 cm^2 (20 × 30 cm).

Table 2. Primer mixes and primer pairs used, plus references to published sequences

Primer mix	Primer pairs	Reference for sequences
PM1-A	Ca1b6	Arioli, Jakob & Reyer (2010)
	RlCA1b5	Garner *et al.* (2000)
	Ga1a19redesigned	Arioli, Jakob & Reyer (2010)
	Rrid064A	Christiansen & Reyer (2009)
	RlCA5	Garner *et al.* (2000)
PM2-A	Res22	Zeisset, Rowe & Beebee (2000)
	ReGa1a23	Christiansen & Reyer (2009)
	Rrid169A	Christiansen & Reyer (2009)
	Rrid013A	Hotz *et al.* (2001)
	Rrid059Aredesigned	Christiansen & Reyer (2009)
PM1-B	Res16	Zeisset, Rowe & Beebee (2000)
	Res20	Zeisset, Rowe & Beebee (2000)
	RlCA2a34	Christiansen & Reyer (2009)
	Re2CAGA3	Arioli, Jakob & Reyer (2010)
PM2-B	Re1CAGA10	Arioli, Jakob & Reyer (2010)
	RlCA1a27	Christiansen & Reyer (2009)
	RlCA18	Garner *et al.* (2000)
	Rrid135A	Christiansen & Reyer (2009)

After 2 days, unfertilized eggs and/or aborted embryos were carefully removed every 2 days to avoid bacterial and fungal development. After 15 days, embryos had reached the free-swimming tadpole stage (Gosner stage 23–25; Gosner, 1960).

EXPERIMENTAL DESIGN

The crossing procedure described previously allows fertilization of eggs from different females with sperm from the same male and, conversely, fertilization of eggs from the same female with sperm from different males. In this way, one can produce half-sib offspring cohorts within and between populations. In our study, we crossed males and females of different origin both within and between genotypes and locations, respectively. Originally, we had planned a fully crossed design with three replicates for all combinations. However, due to insufficient egg numbers in some females and/or failed fertilization through sperm of some males, only the 57 crosses shown in Table 3 could be performed. Although there are some gaps, the design is complete in the sense that all adult genotype × location combinations are represented. Thus, the conditions for testing how offspring performance is affected by type and origin of the parents are fulfilled. All crosses involved at least one hybrid parent. We did not perform crosses between parental males and females. Given the above-mentioned shortage of eggs and/or failed fertilization through male sperm, inclusion of such crosses would inevitably have led to gaps and reduced the number of replicates for crosses involving one or two hybrids. And offspring from these crosses are more relevant for our questions than offspring resulting from crosses between the two parental species.

REARING OF TADPOLES

After reaching the free-swimming stage, groups of five healthy looking tadpoles from the same cross were transferred to 5 litres tubs containing 3·5 L of aged tap water. Additional tadpoles (usually 25 per cross) were used for determining their genotypes and the gamete types produced by the crossed males and females but not further considered in this study. The rearing tubs were placed on four layers of shelves in two climate chambers, one set to 19 °C and the other to 25 °C (±1 °C). Due to the cooling effect of evaporation, this resulted in water temperatures of 18 and 24 °C, respectively. The climate chambers (SR Kältetechnik & Partner) were illuminated by lamps (tulux, 18W/230V/50 Hz) from 6 am to 9 pm (15L:9D regime). Initial arrangement of the boxes on the shelves and weekly changes of their locations were made using the randomizing function in Excel. As performing all crosses took 4 days (2 June 2009–5 June 2009) and hence tadpoles differed in age, their transfer to the climate chamber was staggered correspondingly, so that all tadpoles entered the temperature treatment at the same age.

Tadpoles were fed once a week with a powder mix consisting of 4 parts rabbit food (plant material) and 1 part *Spirulina* tabs (vitamins and algae). Food was provided using a custom-made spoon containing a mean of 0·0114 g (±0·0016 g). Feeding was adjusted to the number of tadpoles still alive in a tub by adding one spoon of food per tadpole. Following some mortality after 6 weeks (11 dead tadpoles out of 335 at 18 °C and 9 dead ones of 320 at 24 °C), the feeding schedule was increased to two times a week. Water was changed every 3 days, with the transfer date as the reference point. We always used aged tap water that had been equilibrated to the room temperature of the respective treatments.

STATISTICAL ANALYSES

We recorded three parameters that are frequently used for describing tadpole performance: days to metamorphosis, survival to metamorphosis and weight at metamorphosis. Metamorphosis was defined as emergence of at least one forelimb (stage 42; Gosner 1960). The number of days from fertilization to this stage was used as a measure for days to metamorphosis, and the number of tadpoles reaching this stage was used to calculate survival to metamorphosis. Tadpoles that had survived but not yet metamorphosed when the experiment was terminated 169 days after fertilization were considered nonsurvivors. The best estimate for body size at metamorphosis is the weight at tail resorption (Travis 1980, 1984). Therefore, metamorphs were held separately in Petri dishes containing humidified cotton until tail resorption was complete and then weighed to the nearest 0·1 mg.

Prior to analysis, the three fitness parameters were tested for their distribution using the Kolmogorov–Smirnov one sample test with Lilliefors modification. As variables were not normally distributed and in order to increase additivity of effects and equality of variance (Snedecor & Cochran 1980) days to and weight at metamorphosis were logarithmically transformed and survival rate was transformed by the arcsine of the square root.

General linear models (GLM) were used to relate these three parameters to the following four factors: two classes of experimentally manipulated temperatures [18 and 24 °C], two parent origins [same population (S), different populations (D)], two parental combinations [both hybrids (H-H), one hybrid and one parental species (H-P)] and five offspring genotypes [LL, LLR, LR, LRR, RR], respectively, two categories, hybrids [LLR, LR, LRR] and parentals [LL, RR]. Two-way interactions between the four factors were also included in the model. Factors with significant effects were subsequently subjected to pairwise comparisons using Scheffe's test. All tests were performed using Systat 11.0 (Systat Software Inc., Chicago, IL, USA).

A few crosses resulted in mixed offspring genotypes (e.g. LR and LLR or LL and LLL) because some adults produced two gamete types (cf. Table 3). These mixed cohorts were included in the GLM with two offspring categories, but not in the one with the five genotypes. Our crosses also generated a few autotriploids (LLL and RRR) that occurred in mixed cohorts with mostly diploid larvae of the parental species (LL and RR). Although there is some debate whether performance of autotriploids differs from that of autodiploids (e.g. Stebbins 1985; Parisod, Holderegger & Brochmann 2010) pooling of LLL and RRR with LL and RR could not bias our results, because there were only three autopolyploid tadpoles in our rearing experiment: one RRR in cross female 015-68 × male 014-48 at 18 °C, one LLL in cross 014-62 × 015-50 at 18 °C and one LLL in cross 014-21 × 014-56 at 24 °C (see Table 3). None of those three individuals reached metamorphosis before the end of the experiment. So they were not included in the analyses of days to and weight at metamorphosis and must have had a negligible impact on survival values.

Results

GAMETE AND OFFSPRING TYPES

The artificial crosses produced five types of pure offspring cohorts (LL, LLR, LR, LRR and RR) and four types of mixed cohorts (LR/LLR, LR/LRR, LL/LLL and RR/RRR). Combined with the known genotypes of the crossed adults, this allows identification of the gametes produced by males and females (Table 3). In males, all individuals produced exclusively haploid gametes, independent of their genotype and origin. Female gamete production was more diverse and varied with both genotype and locality. In Kyritz, all triploid female hybrids produced the expected haploid gametes, namely L eggs in LLR and R eggs in

Table 3. Design for artificial crosses between females (vertical) from three populations and males (horizontal) from two populations.

Population	Indiv. numb.	Genotype	Gametes	Kyritz (DE) 014-05 **LR** *R*	014-48 **LR** *R*	014-59 **LLR** *L*	014-56 **LLR** *L*	014-55 **LLR** *L*	014-49 **LRR** *R*	014-11 **LRR** *R*	014-58 **LRR** *R*	Sastin-Strace (SK) 015-06 **LR** *R*	015-03 **LR** *R*	015-04 **LR** *R*	015-50 **LL** *L*	015-47 **LL** *L*	016-23 **RR** *R*	016-21 **RR** *R*	016-22 **RR** *R*
Kyritz (DE)	014-25	**LR**	*LR*	**LRR**		**LLR**			**LRR**			**LRR**			**LLR**		**LRR**		
	014-63	**LR**	*LR*					**LLR**			**LRR**			**LRR**				**LRR**	
	014-62	**LLR**	*L+LL*	**LR/LLR**		**LL**/(LLL)			**LR/LLR**			**LR/LLR**			**LL/LLL**		**LR**/(LLR)		
	014-21	**LLR**	*L+LL*				**LL/LLL**			**LR**/(LLR)			**LR/LLR**						
	014-24	**LRR**	*R*	**RR**		**LR**			**RR**			**RR**			**LR**		**RR**		
	014-67	**LRR**	*R*		**RR**		**LR**			**RR**									
	014-26	**LRR**	*R*					**LR**						**RR**		**LR**		**RR**	
Sastin-Strace (SK)	015-72	**LR**	*R*	**RR**		**LR**			**RR**			**RR**			**LR**		**RR**		
	015-73	**LR**	*R*		**RR**		**LR**			**RR**									**RR**
	016-49	**LL**	*L*		**LR**		**LL**				**LR**			**LR**					
	015-64	**LL**	*L*																
	015-68	**RR**	*R+RR*	**RR**/(RRR)	**RR/RRR**		**LR/LRR**		**RR**/(RRR)			**RR**/(RRR)							
	015-71	**RR**	*R*								**RR**			**RR**					
Hellberg (CH)	017-44	**LL**	*L*	**LR**		**LL**			**LR**			**LR**							
	017-48	**LL**	*L*																

Bold letters indicate the genotypes of the individuals, italic letters the gametes they produced. The background colour shows whether crossings were between females and males from the same (grey) or from different populations (white). For those crosses that produced fertilized eggs, the genotypes of the resulting offspring are shown in the main body of the table. In cases where a female × male combination resulted in offspring cohorts with two different genotypes, the more frequent one is listed first, but only genotypes printed in bold were included in the rearing experiment. All crosses involved at least one hybrid. For further details on the design see Material and Methods.

Table 4. Results from six general linear models analyses, two each for the three dependent variables survival, days to metamorphosis and weight at metamorphosis

	Survival			Days to metamorphosis			Weight at metamorphosis		
Source	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Temperature	1/1	12·70/30·20	**0·001/0·000**	1/1	81·04/156·91	**0·000/0·000**	1/1	8·02/18·60	**0·007/0·000**
Offspring type	4/1	11·48/39·33	**0·000/0·000**	4/1	3·39/8·80	**0·015/0·004**	4/1	6·12/7·11	**0·000/0·010**
Population type	1/1	0·69/0·91	0·410/0·342	1/1	0·96/0·36	0·331/0·551	1/1	0·02/0·22	0·896/0·643
Parent combination	1/1	4·06/6·05	**0·047/0·016**	1/1	1·97/0·57	0·166/0·425	1/1	0·00/0·82	0·989/0·368
Offspring type × Temp.	4/1	1·12/0·01	0·355/0·938	4/1	2·90/3·73	**0·029/0·057**	4/1	2·83/6·66	**0·034/0·012**
Population type × Temp.	1/1	1·89/1·12	9·173/0·268	1/1	1·03/2·52	0·314/0·116	1/1	1·06/0·02	0·308/0·895
Parent comb. × Temp.	1/1	1·44/0·80	0·234/0·374	1/1	0·28/0·07	0·599/0·799	1/1	0·40/0·18	0·531/0·676
Offspr. type × Popul. type	4/1	1·83/2·97	0·131/0·088	4/1	1·82/0·96	0·137/0·329	4/1	2·00/0·00	0·110/0·981
Parent comb. × Popul. type	1/1	0·01/0·02	0·915/0·894	1/1	0·61/0·09	0·437/0·767	1/1	0·01/0·30	0·924/0·588
Remaining tadpoles	1/1	26·48/23·79	**0·000/0·000**						
Error	81/99			60/77			49/64		

Values in front of the/refer to analyses with five offspring genotypes (LL, LLR, LR, LRR and RR); values behind the/are from analyses with two offspring categories: hybrids (LLR, LR and LRR pooled) and parental species (LL and RR pooled plus two LLL and one RRR in the survival data set). Significant results are shown in bold. Three-way interactions and one-two-way interaction of sources (offspring type × parental combination) could not be included in the analyses because of missing values.

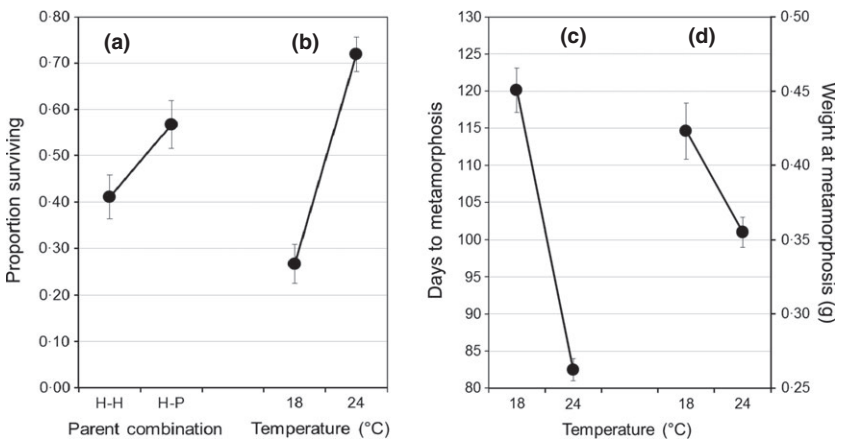

Fig. 2. Proportions of surviving tadpoles in relation to parent combination (a) and temperature (b), and days to metamorphosis (c) and weight at metamorphosis (d) in relation to temperature. Shown are means with standard errors. H-H: both parents are hybrids, H-P: one parent is a hybrid (LLR, LR or LRR) and the other from a parental species (LL or RR).

LRR individuals; but LLR females also produced a small number of diploid LL eggs (average of 13·5%). Among the diploid female hybrids, those from Kyritz produced diploid LR eggs, while those from Šaštín-Stráže produced haploid R eggs. All females of the two parental species produced the expected haploid eggs, but one RR female from Šaštín-Stráže produced also a few diploid RR eggs (average of 1·5%).

TADPOLE PERFORMANCE

Table 4 shows the results from the three GLM analyses relating offspring survival, days to metamorphosis and weight at metamorphosis to the four experimentally manipulated factors and their two-way interactions. The most consistent significant effects on tadpole performance were exerted by temperature and offspring genotype. At 24 °C, survival was significantly higher, time to metamorphosis was shorter and weight at metamorphosis was lower than at 18 °C (Table 4, Figs. 2b-d). Survival was lowest for offspring with the two parental genotypes (LL, RR) and highest for those of the three pure hybrid cohorts (LR, LLR, LRR) (Fig. 3a). At 18 °C, the pattern for days to and weight at metamorphosis was basically a mirror image of the survival pattern: highest values for LL and RR and lowest values for LR, LLR and LRR (Fig. 3b,c). Pairwise comparisons revealed no significant differences between the two parental species and the three hybrid types, respectively, for any of the three performance measures. When results from cohorts of the three pure hybrid types and the two parental species are pooled into two categories (right sides of Fig. 3a–c), differences between the hybrids and parental species are significant for survival. For days to, and weight at, metamorphosis they are significant at 18 °C, but not at 24 °C; hence the offspring type × temperature interactions for these two variables (Table 4). For days to metamorphosis, pairwise tests of this interaction showed significant differences between developmental rate at 18 and 24 °C for the three diploid genotypes (LL, LR, RR), but not for the two triploid ones (LLR, LRR).

As tadpoles that had not metamorphosed until the end of the experiment (day 169) were considered nonsurvivors,

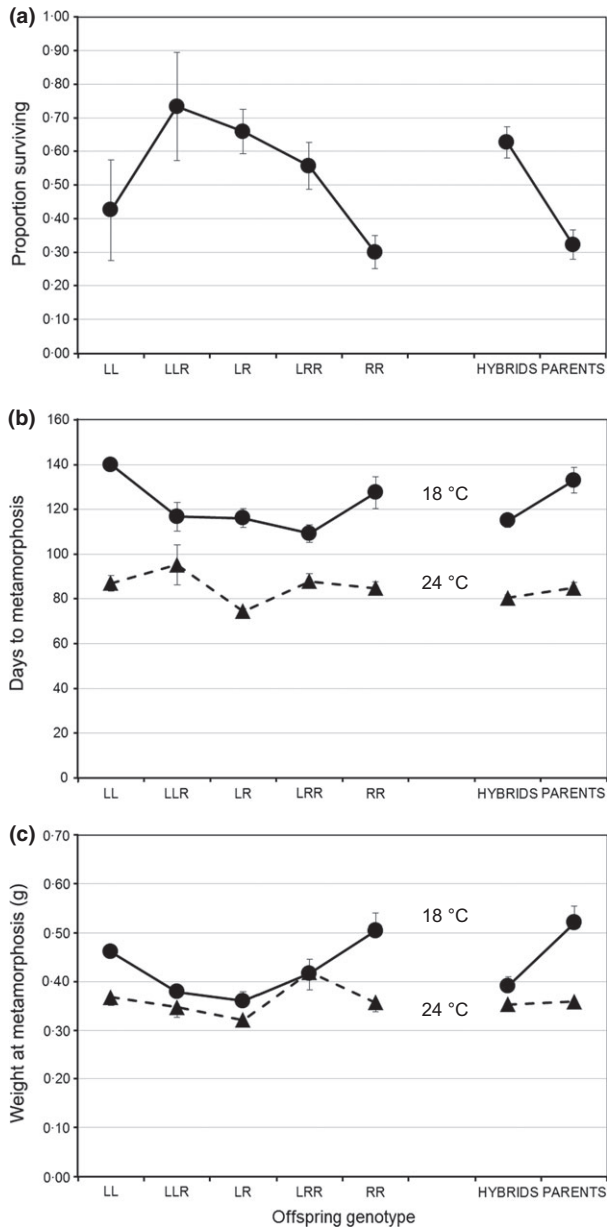

Fig. 3. Proportions of surviving tadpoles (a), days to metamorphosis (b) and weight at metamorphosis (c) in relation to offspring genotype. Shown are means with standard errors. On the left side of each figure, the five categories of genotypes are plotted separately; on the right side, they are grouped into two categories: hybrids (LLR, LR and LRR pooled) and parents (LL and RR pooled, including two LLL and one RRR in Fig. 3a). In figures b) and c), results are also separated by temperature, because of the significant genotype × temperature interaction (see Table 4).

survival values are potentially confounded by long development times. This is supported by the significant relationship between survival and the number of tadpoles remaining at the end of the experiment (Table 4). However, the above-mentioned effects of temperature and offspring type on survival emerged, even though tadpole number was included in the analysis. Survival, but not time to and weight at metamorphosis, was also influenced by the combination of the parents. Survival of offspring from crosses between hybrid males and females was significantly lower than survival of offspring from crosses where only one parent was a hybrid and the other belonged to a parental species (Fig. 2a). Crosses within and between populations (population type) produced no significant differences for any of the three performance variables, nor did the two-way interactions, with the exception of the above-mentioned offspring type × temperature interaction.

Discussion

The results of this study show that development of water frog tadpoles is affected by both ecological factors (here represented by temperature) and genetics (here represented by genotypes of parents and offspring). Both factors influenced all three fitness parameters: survival rate, days to metamorphosis and weight at metamorphosis. Overall, hybrids performed better than the parental species. It seems plausible to assume that this 'hybrid vigour' results from spontaneous heterosis that is due to genetic mechanisms (reviewed by Lippmann & Zamir 2007), like the suppression of deleterious alleles in one parental genome through dominant alleles in the other (dominance hypothesis), a combination of alleles that are particularly advantageous in the heterozygous state (overdominance hypothesis) and/or modification of genes by those at other loci (epistasis). However, evidence for heterosis in clonal and hemiclonal hybrids is mixed. Support for spontaneous heterosis comes from crossing experiments of Hotz *et al.* (1999) who found better survival, higher growth rate and shorter time to metamorphosis in offspring of F-1 *P. esculentus* than in those of the two parental species. However, other studies on the same system did not detect heterosis effects with respect to growth, development, oxygen requirement, heat resistance and parasite infection (Plenet, Hervant & Joly 2000; Plenet *et al.* 2005; Litvinchuk *et al.* 2007; Planade *et al.* 2009) nor was spontaneous heterosis found in newly synthesized strains of the unisexual fish *Poeciliopsis* (Wetherington, Kotora & Vrijenhoek 1987). Thus, most studies seem to indicate that heterosis alone is usually not sufficient to explain the (hemi)clonal hybrids' ecological success. It may, however, operate in conjunction with other mechanisms, such as habitat segregation and/or selection of the fittest clones from a spectrum of genotypes that arose via multiple hybridization events (Hotz *et al.* 1999; Plenet *et al.* 2005; Planade *et al.* 2009). Because of this synergy, heterosis effects will be modified by the environment (Lippmann & Zamir 2007). Hence, they may show up under certain ecological conditions and for some traits, but not in other circumstances.

Therefore, we below discuss the specific results for the three fitness parameters one by one in relation to temperature and genotype. At the end, we outline potential consequences for population composition.

SURVIVAL RATE

Survival rate was much higher at 24 °C than at 18 °C (Fig. 2b) and higher in hybrid tadpoles than in those of the parental type, but not different between genotypes within these two offspring categories, that is not between LL and RR (including two LLL and one RRR), respectively, LLR, LR and LR. The absolute survival values may, to some extent, have been confounded by long development times, because tadpoles that had survived but not yet metamorphosed when the experiment was terminated 169 days after fertilization were considered nonsurvivors. However, the risk of such a bias is probably negligible, because our experimental period was much longer than usual development times (e.g. Semlitsch, Hotz & Guex 1997) and the remaining tadpoles will have died anyway. Moreover, to avoid this potential bias, we included the number of tadpoles surviving at the end of the experiment as a covariate into the GLM for survival and, yet, the temperature and genotype effects emerged. Thus, survival differences between temperatures and offspring types are real. Better larval survival at high than at low temperature has been reported for water frogs before (Orizaola & Laurila 2009); but a direct effect of low temperature on mortality does not seem to be widespread in experimental studies of amphibian development. Under natural conditions, however, there often will be an indirect effect via prolonged time to metamorphosis and, hence, extended exposure to aquatic predators and/or risk of pond desiccation.

Results of Negovetic *et al.* (2001) indicate that LL may be better adapted to warm and LR to cold temperatures. Under laboratory conditions, tadpole survival for the parental species *P. lessonae* (LL) was better at 24 °C, whereas that of diploid hybrid *P. esculentus* (LR) was better at 18 °C. These results were corroborated by the distribution in natural ponds: the proportion of hybrids increased with decreasing water temperature. The authors suggest that this thermal niche differentiation may help parentals and hybrids to coexist, despite of many genetic, ecological and morphological similarities. In our study, we found no offspring type × temperature interaction on survival (Table 4), indicating that temperature affected survival of all genotypes equally. The difference between the results from the two studies may partly be related to differences in larval periods that were much longer in our study. Maybe increasing general mortality late in the larval period has obliterated species differences that may have existed earlier.

The reasons for the overall lower survival of parental offspring types in our study also remain unclear. The usual explanation assumes that high parental type mortality results from the fusion of two clonal hybrid genomes (cf. Table 1) with the same fixed recessive deleterious mutations (Muller 1964; Vorburger 2001a). In our experiment, however, this could be true for only one out of the 26 cohorts with offspring of the parental types (21 RR, 5 LL; see Table 3) and, thus, cannot explain the high mortality of the parental types. In all other cases, LL and RR tadpoles originated from crossings between males and females from far apart populations with different hemiclones (e.g. female 015-72 × male 014-05) and/or from pairs where one or both parents had recombined the genome prior to gametogenesis (e.g. female 016-49 × male 014-56). This introduces heterozygosity into LL and RR tadpoles through the combination of either different hemiclones or one clonal and one sexual genome. As shown by Vorburger (2001a,b) and Guex, Hotz & Semlitsch (2002), such heterozygosity is sufficient to overcome the effect of deleterious mutations on the clonally transmitted genomes. Even in the one intrapopulation cross from Šaštín-Stráže where two clonal genomes were combined (female 015-72 × male 015-06 in Table 3), these genomes must not necessarily have been identical with respect to their deleterious mutations. Given that in this population LR hybrids live in sympatry with both parental species primary hybridization, as well as backcrossing between hybrids and parental species, is likely to happen fairly regularly (N.B. M. Pruvost *et al.* in prep.). Backcrossing will lead to recombination of originally clonal R genomes from LR hybrids once they have arrived in sexual RR individuals, whereas repeated primary hybridization will result in different and relatively young hemiclones that are unlikely to have already accumulated many deleterious mutations on the same loci.

Yet, low genetic diversity and/or genome incompatibility may have played a role in our study. This is indicated by the fact that offspring survival was lower in crosses where both parents were hybrids (H-H) than in those where one parent was from a parental species (H-P).

DAYS TO METAMORPHOSIS

The faster larval development at high compared with low temperatures found in this study is typical for amphibian species. The pattern has repeatedly been demonstrated in experiments like ours, where tadpoles were raised under different temperature regimes (e.g. Alvarez & Nicieza 2002; Walsh, Downie & Monaghan 2008). It also emerges from several studies where time to metamorphosis was found to decrease when pond temperature increased as a result of experimentally lowered water levels (e.g. Loman 1999) or decreasing canopy cover (e.g. Skelly, Freidenburg & Kiesecker 2002; Hocking & Semlitsch 2008; Van Buskirk 2011).

In contrast to survival, the effect of temperature on days to metamorphosis varied between offspring genotypes, as indicated by the significant genotype × temperature treatment (Table 4). Overall, the hybrids develop faster than the parental species at 18 °C, whereas at 24 °C the two categories do not differ (Fig. 3b, right). Pairwise tests on this interaction revealed that the difference between the two temperatures is more pronounced for the diploid offspring forms (LL, RR and LR) than for the triploid ones (LLR, LRR). This suggests that decreasing temperatures

affect the development controlling mechanisms in triploids less than in diploids. As a result, triploids may be better adapted to develop under cold conditions than diploids, a pattern that has been found in numerous species, including others frogs (Dufresne & Hebert 1998; Lencioni 2004; Otto *et al*. 2007). It is also in line with the high proportion of polyploid plants and animals found under the harsh environmental conditions at high latitudes and altitudes and at the geographical periphery of species ranges. The explanation for the higher temperature tolerance seems to lie in the additional genome and the resulting increased levels of somatic heterozygosity which, in turn, leads to changes in many morphological and physiological traits, including larger cell and body size and more enzyme varieties (Otto & Whitton 2000; Mable 2004).

WEIGHT AT METAMORPHOSIS

The factors that had a significant influence on the weight at metamorphosis were the same as the ones affecting days to metamorphosis: temperature, offspring genotype and their interaction (Table 4). This is not surprising because – all other things being equal – development time directly affects time for feeding and, hence, growth. As a result, we can expect a positive correlation between time to and weight at metamorphosis. This has been found in numerous studies on amphibian larval development (e.g. Semlitsch 1993; Tejedo *et al*. 2010) including this study. The overall lower weight at 24 °C than at 18 °C (Fig. 2d) can be explained by the shorter development time under warm conditions (Fig. 2c), and the weight differences between hybrids and parental species are also related to their respective development times (Figs. 3b,c): faster hybrid tadpole development at 18 °C leads to lower weight at metamorphosis, whereas similar developmental rate at 24 °C does not result in weight difference between the two groups. Interestingly, at 24 °C (but not at 18 °C) LRR and RR offspring tended to be heavier than LL, LR and LLR. These two heavier groups have an excess of R over L genomes (ratios $\geq 0{\cdot}67$), whereas the three lighter groups have not (ratios $\leq 0{\cdot}5$). Increasing size with increasing R:L genome ratios has also been found among hybrid larvae (LRR, LR, LLR) in natural ponds (Jakob 2007). The differences are likely to reflect a weight effect of the *P. ridibundus* genome, that is the genome of the largest species in the water frog complex. According to our present results, the extent of this effect may vary with temperature.

CONSEQUENCES FOR POPULATION STRUCTURE AND DYNAMICS

Larval anurans exhibit high levels of phenotypic plasticity in life-history traits; and survival rates, time to and age at metamorphosis vary markedly among species and with the specific combination of various abiotic and biotic factors (Stahlberg, Olsson & Uller 2001; Van Buskirk & Arioli 2005; Lindgren & Laurila 2009; Van Buskirk 2009, 2011). The resulting multitude of species × environment interactions makes identification of the most important determinants of fitness difficult. However, genotype and temperature are definitely very important factors. They have repeatedly been shown to affect larval performance and this will influence the structure and dynamics of populations directly and indirectly (Hellriegel 2000; Hellriegel & Reyer 2000).

Direct effects arise from differences in developmental rates at the two temperatures. Given that at 18 °C (but not at 24 °C) hybrid larvae metamorphose sooner than those of the parental species (Fig. 3b), this will improve their survival under cold conditions in two ways. First, they are exposed to aquatic predators (the major cause of tadpole mortality) for shorter times than larvae of the parental species. Second, after entering their terrestrial habitat, early metamorphosing individuals survive better (Altwegg & Reyer 2003). Such selective advantage of hybrids under cold conditions has also been shown in other studies on larval and adult *Pelophylax* (Negovetic *et al*. 2001; Anholt *et al*. 2003).

Indirect effects on the structure and dynamics of populations arise from reinforcement of the temperature-related survival differences via the mating pattern and genetic effects. Given that mating between LR, LLR and LRR seems to be random (Günther & Plötner 1989; Som & Reyer 2006; B. Rondinelli, unpubl. data) an increasing number of hybrids will result in a higher proportion of matings between them. About half of these hybrid-hybrid matings (H-H) result in the parental genotypes LL and RR (Table 1), and offspring from H-H combinations survive worse than those from H-P combinations where one partner is from a parental species (Fig. 2a). As a result of these direct and indirect effects, cold temperatures will put *P. lessonae* and *P. ridibundus* at a selective disadvantage, compared with *P. esculentus* so that the parental species will gradually be diluted from mixed parental-hybrid populations, and all-hybrid populations will emerge and persist.

Thus, our finding that under cold conditions *P. esculentus* hybrids in general and triploids in particular are at a selective advantage compared with the parental species *P. lessonae* and *P. ridibundus* offers a new explanation for the observed geographic distribution of different breeding system. The predominance of all-hybrid populations in the cooler northern range of the water frog distribution – for example in Sweden, Denmark, northern Germany and northern Poland (Plötner 2005) – seems at least in part a result of direct and indirect temperature effects. However, the reasons why many such all-hybrid populations differ markedly in the relative numbers of male and female LR, LLR and LRR is not yet fully understood; but differences in gamete production patterns combined with several abiotic and biotic environmental factors offer the most likely explanation (Christiansen 2009; Christiansen *et al*. 2010; Jakob, Arioli & Reyer 2010; Christiansen & Reyer 2011).

Acknowledgements

We are grateful to Peter Mikulíček and Matje Kautman for their help in catching frogs in Slovakia, to Sandra Röthlisberger for assisting with the laboratory analyses, to Ursina Tobler for helpful discussions and three anonymous reviewers for valuable comments on the manuscript. Catching of frogs was carried out based on permits of our colleagues in Germany and Slovakia. Import to Switzerland was permitted by the Bundesamt für Veterinärwesen (Abteilung Import/Export). Keeping of frogs in captivity for observations and experiments was granted by the Kantonales Veterinäramt, Zurich. The study was funded by the Swiss National Science Foundation through a grant to H.-U. Reyer (No. 3100A0-120225/1).

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Received 14 June 2012; accepted 26 November 2012
Handling Editor: Robbie Wilson