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FIXATION OF DELETERIOUS MUTATIONS IN CLONAL LINEAGES: EVIDENCE FROM HYBRIDOGENETIC FROGS

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Abstract.—The hemiclonal waterfrog *Rana esculenta* (RL genotype), a bisexual hybrid between *R. ridibunda* (RR) and *R. lessonae* (LL), eliminates the L genome from its germline and clonally transmits the R genome (hybridogenesis). Matings between hybrids produce *R. ridibunda* offspring, but they generally die at an early larval stage. Mortality may be due to fixed recessive deleterious mutations in the clonally inherited R genomes that were either acquired through the advance of Muller's ratchet or else frozen in these genomes at hemiclone formation. From this hypothesis results a straightforward prediction: Matings between different hemiclones, that is, between *R. esculenta* possessing different R genomes of independent origin, should produce viable *R. ridibunda* offspring because it is unlikely that different clonal lineages have become fixed for the same mutations. I tested this prediction by comparing survival and larval performance of tadpoles from within- and between-population crossings using *R. esculenta* from Seseglio (Se) in southern, Alpnach (Al) in central, and Elliker Auen (El) in northern Switzerland, respectively. Se is isolated from the other populations by the Alps. Enzyme electrophoresis revealed that parents from Se belonged to a single hemiclone that was different from all hemiclones found north of the Alps. Parents from Al also belonged to one hemiclone, but parents from El belonged to three hemiclones, one of which was indistinguishable from the one in Al. *Rana esculenta* from Se produced inviable tadpoles when crossed with other hybrids of their own population, but when crossed with *R. esculenta* from Al and El, tadpoles successfully completed metamorphosis, supporting the hypothesis I tested. Within-population crosses from Al were also inviable, but some within-population crosses from El, where three hemiclones were present, produced viable offspring. Only part of the crosses between Al and El were viable, but there was no consistent relationship between hemiclone combination and tadpole survival. When backcrossed with the parental species *R. ridibunda*, hybrids from all source populations produced viable offspring. Performance of these tadpoles with a sexual and a clonal genome was comparable to that of normal, sexually produced *R. ridibunda* tadpoles. Thus, in the heterozygous state, the deleterious mutations on the clonal R genomes did not appear to reduce tadpole fitness.

Key words.—Clonal reproduction, crossing experiment, deleterious mutations, fixation, hybridogenesis, Muller's ratchet, *Rana esculenta* complex.

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Spontaneous mutations provide the raw material for adaptive evolution of organisms, but only a small fraction of new mutations are beneficial. Most mutations are either neutral or deleterious (Simmons and Crow 1977; Crow 1993). There is increasing evidence that new deleterious mutations arise at a substantial rate, possibly as high as one per genome per generation (Drake et al. 1998; Kondrashov 1998; Lynch et al. 1999). The need to mitigate the damaging effects of deleterious mutations may at least in part explain several important biological phenomena (reviewed by Charlesworth and Charlesworth 1998), including the evolution of diploidy; the evolution of inbreeding avoidance or, more generally, mate choice for good genes; the evolution of degenerate Y chromosomes and dosage compensation; and, most importantly, the evolution and maintenance of sexual reproduction and genetic recombination.

Muller (1964) was the first to realize the consequences that deleterious mutations can have for clonal populations. Due to recombination, offspring in a sexual population can have fewer deleterious mutations than either parent, whereas those in a clonal population have all the mutations of their parent and possibly new mutations occurring in that generation. As soon as every individual in a clonal population carries at least one mutation, the mutation-free class is lost and cannot be regenerated, assuming there is no back mutation. The new least-loaded class eventually meets the same fate and the process continues, leading to a decline in mean population fitness. Felsenstein (1974) named this process Muller's ratchet. Recombination reduces the rate of accumulation of deleterious mutations and may provide an important advantage for sexual over clonal reproduction.

Therefore, Muller's ratchet has attracted considerable theoretical interest. Mutation accumulation can be modeled by subdividing the population into classes based on the number of mutations. Following one generation of mutation and selection, individuals are randomly sampled from these classes to generate the set of mutational classes in the next generation (e.g., Haigh 1978; Stephan et al. 1993). However, such models do not consider the fate of mutant alleles at individual loci. As pointed out by Charlesworth et al. (1993), Muller's ratchet may not only lead to the accumulation of deleterious mutations but also to their fixation in clonal populations. Recent simulation studies of Muller's ratchet therefore have modeled genomes with finite numbers of loci and followed the frequency of mutant alleles over time (Higgs and Woodcock 1995; Charlesworth and Charlesworth 1997). It was found that clonal populations indeed suffer from the fixation of deleterious mutations and that in the haploid case, the rate of fixation equals both the rate at which the mean number of mutations per haploid genome increases and the rate at which the least loaded classes are lost from the population. This finding is of great biological importance and has been invoked to explain, for example, the rapid degeneration of incipient Y chromosomes or neo-Y chromosomes (Charlesworth and Charlesworth 1997, 1998; Gordo and Charlesworth 2000). How these fixations occur is best understood by con-

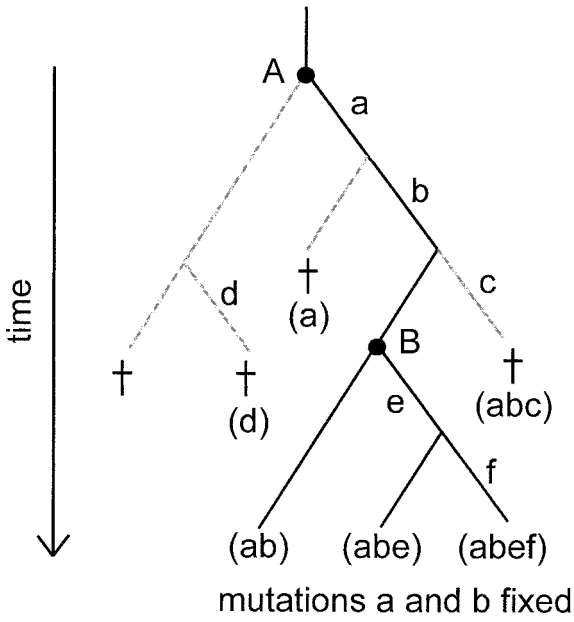

FIG. 1. Schematic representation of a genealogical tree of an asexual population along a time axis (modified from Higgs and Woodcock 1995). Lowercase letters represent mutations. As the most recent common ancestor of the extant population shifts from individual A to individual B, mutations a and b become fixed in the population.

sidering a hypothetical genealogical tree of an asexual population (Higgs and Woodcock 1995; Fig. 1). Starting from a single individual (A), the population splits due to mutations into separate branches. Random genetic drift causes some of these branches to die out and after a certain time, the most recent common ancestor of the extant population will shift to individual B, resulting in the fixation of all mutations that arose on the line of descent from A to B.

European waterfrogs of the *Rana esculenta* complex provide an excellent system to look for evidence of this process. The hemiclonal frog *R. esculenta* (RL genotype) is a diploid bisexual hybrid between the two parental species *R. ridibunda* (RR) and *R. lessonae* (LL; Berger 1967, 1968). Hybrids reproduce by hybridogenesis (Schultz 1969; Tunner 1974), that is, they exclude premeiotically one of their parental genomes from the germline and produce functional haploid gametes containing the unrecombined (clonally transmitted) genome of only one parental species (reviewed by Graf and Polls Pelaz 1989). Over large parts of central Europe, including my study sites in Switzerland, *R. esculenta* exclusively coexists with one of its parental species, *R. lessonae*, forming the so-called L-E system (Uzzell and Berger 1975). In these populations, *R. esculenta* only transmits the R genome in its gametes (Tunner and Heppich-Tunner 1991). It is assumed that *R. esculenta* has immigrated from eastern European areas of sympatry between *R. ridibunda* and *R. lessonae* after the last glacial period (Wuerm age), approximately 10,000 years ago. Assuming a mean generation time of two years, R genomes in hybridogenetic *R. esculenta* may have been transmitted clonally for roughly 5000 generations. However, some clonal R genomes may be younger, because immigration was permanently possible after the last glaciation, or even older, if ancient clonal lineages were already present in refugial areas of *R. lessonae* during the Wuerm age (H. Hotz, pers. comm.).

In the L-E system, *R. esculenta* acts as a sexual parasite of *R. lessonae*, because *R. esculenta* can only persist by mating with *R. lessonae*. Such matings produce *R. esculenta* offspring that again exclude the L genome from their germline. These offspring are hemiclonal because they possess a clonally transmitted R genome and a sexually transmitted, genetically variable L genome. Matings between hybrids also occur and produce *R. ridibunda* offspring, but those generally die at an early larval stage (Blankenhorn et al. 1971; Heusser and Blankenhorn 1973; Berger and Uzzell 1977; Binkert et al. 1982; Semlitsch and Reyer 1992; Vorburger 2001a). Several authors have hypothesized that the lethality of offspring from *R. esculenta* × *R. esculenta* matings might be due to fixed recessive deleterious mutations in the hybrid's R genomes (Berger 1976; Graf and Müller 1979; Graf and Polls Pelaz 1989; G.-D. Guex, H. Hotz, and R. D. Semlitsch, unpubl. ms.). In *R. esculenta*, these mutations would be permanently in the heterozygous state and therefore be sheltered from selection, but they would become homozygous in *R. ridibunda* offspring from *R. esculenta* × *R. esculenta* matings. From this hypothesis results a straightforward prediction: matings between different hemiclones, that is, between *R. esculenta* possessing evolutionarily independent R genomes of different origin by hybridization, should produce viable offspring because it is unlikely that different clonal lineages have fixed the same mutations. I tested this prediction using experimental crossings. I collected *R. esculenta* from three widely spaced populations likely to contain different hemiclones and compared the survival rates of tadpoles produced by within- and between-population crossings of these frogs. To test whether mutations on clonal R genomes also reduce offspring fitness in the heterozygous state, I additionally backcrossed *R. esculenta* from these populations with their parental species *R. ridibunda*, producing *R. ridibunda* tadpoles with one sexually and one clonally transmitted R genome.

MATERIALS AND METHODS

Source Populations

I obtained *R. esculenta* for crossings from three source populations in Switzerland (Fig. 2). Populations were chosen to be far apart from each other and therefore supposed to contain different hemiclones. Care was taken to select populations still containing native L-E systems, because some areas of Switzerland, where *R. ridibunda* is not native, have been invaded by introduced *R. ridibunda* from eastern Europe and Anatolia (Grossenbacher 1988). It was essential to use native L-E systems because introduced *R. ridibunda* can mate with native *R. esculenta* as well as with native *R. lessonae* and possibly form new hemiclones without a long history of clonal inheritance.

The first population is located in an area called Elliker Auen (El) near Ellikon in northern Kanton Zürich. The breeding habitat of this population is adjacent to the rivers Rhine and Thur and consists of several ponds within ancient river oxbows. The second population is located near Alpnach (Al)

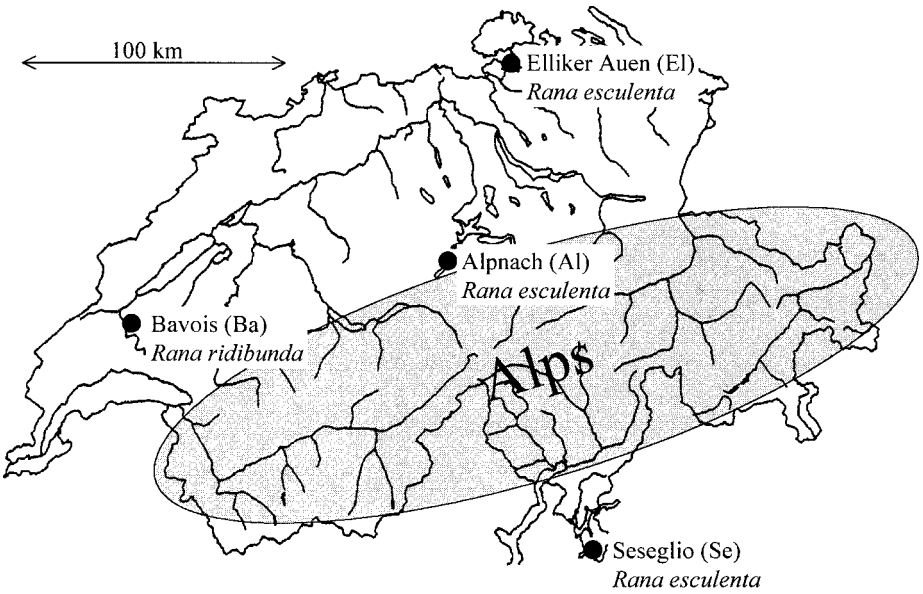

FIG. 2. Map of Switzerland showing the locations of the four source populations of waterfrogs used in this study.

in Kanton Obwalden. It breeds in a single, densely vegetated pond completely surrounded by thin forest. Al lies approximately 80 km southwest from El, but there appear to be no insurmountable geographical barriers between the two locations. The third population is located near Seseglio (Se) in Kanton Tessin. Here waterfrogs breed in a series of five closely adjacent human-made ponds. Se is completely isolated from my other source populations by the Alps, which prevent any natural dispersal by waterfrogs. All frogs were caught by hand at night, on 12 May 1998 in Al, on 16 May in Se, and on 21 May in El. I determined taxon and sex of all frogs and kept five male and five female *R. esculenta* from each population. I brought these frogs to the University of Zürich and stored them in a cool room at 10°C until crossing. A subsample of the remaining frogs was toe-clipped before release into their ponds of origin. Clipped toes were transported on dry ice and stored at −80°C. To obtain a better estimate of the relative frequencies of *R. lessonae* and *R. esculenta* and of the hemiclone composition within *R. esculenta,* I resampled all populations for tissue once or twice in 1999.

As a source for *R. ridibunda,* I chose a population near Bavois (Ba), about 20 km north of Lausanne, Switzerland (Fig. 2). *Rana ridibunda* was introduced in this area around 50 years ago and has replaced the native waterfrogs *R. lessonae* and *R. esculenta* (Grossenbacher 1988). Now *R. ridibunda* forms large, pure populations in several parts of western Switzerland, including the collection site. It can safely be assumed that reproduction in this population was purely sexual for many generations. I captured *R. ridibunda* on 21 May and collected five males and five females that were stored at 10°C.

Crossing Design

I produced five replicate crosses of the 16 possible population combinations in a 4 × 4 factorial breeding design. Using artificial fertilizations (Berger et al. 1994), five females per population were each crossed with four males, one from every population, and five males per population were each crossed with four females, again one from every population, producing at total of 80 half-sibling families. Before crossings, taxon identification of all frogs was confirmed by electrophoresis of lymph albumin (Tunner 1973). All offspring produced from these crosses were *R. ridibunda* (RR genotype), but they either possessed two sexually transmitted R genomes (crosses between two *R. ridibunda*), one sexual and one clonal R genome (crosses between *R. ridibunda* and *R. esculenta*), or two clonal R genomes (crosses between two *R. esculenta*), stemming either from the same or from two different populations.

On 23 May 1998 I injected all females with the fish hormone LH-RH (H-7525, Bachem, Inc., Bubendorf, Switzerland) to induce ovulation. On the same day, I euthanized the males with 3-aminobenzoic acid ethyl ester (MS-222; A-5040, Sigma, Inc., Buchs, Switzerland) and dissected them, removing testes for fertilizations and a tissue sample (leg muscle) for later protein electrophoresis. Testes were stored in Holtfreter's solution at 4°C until use. When females began to ovulate, 24 h later, I prepared sperm suspensions of one male from each of the four populations by crushing both testes in petri dishes containing 15–20 ml of pond water. Then I stripped the eggs of one female into these sperm suspensions, cycling between the males to avoid confounding potential effects of ovulation order with paternal effects. After 5 min I rinsed the four sperm suspensions into new petri dishes and covered the fertilized eggs with fresh pond water. Then the eggs of a female from a different population were stripped into the four sperm suspensions. This procedure was repeated until one female from all four populations was crossed with each of the four males. Subsequently, I prepared four new sperm suspensions for the next round of fertilizations following the same protocol. Five rounds of fertilizations were performed to complete the crossing design. I rotated the order in which females were picked from the four populations between rounds to avoid any bias due to potential

effects of fertilization order (e.g., sperm dilution). All crosses were made on 24 May. The females were then toe-clipped and returned to their populations of origin.

On 25 May, one day after fertilizations, I transferred the eggs from petri dishes into larger containers filled with 1 L of pond water. Containers were stored in a climatized room at a constant temperature of 20°C. On 27 May, when developing embryos had reached approximately tail bud stage (stage 17; Gosner 1960) and were clearly recognizable by eye, I counted the number of undeveloped eggs and developing embryos in every container to obtain an estimate of fertilization rates. This estimate may be confounded with early embryonal mortality, but time constraints prevented me from obtaining a more accurate measure of fertilization success by examining the eggs under a dissection microscope during early cleavage. Tadpoles hatched between 29 May and 31 May. After hatching, I repeatedly removed undeveloped eggs or dead tadpoles from the containers to maintain good water quality and prevent the outbreak of fungal infections. On 6 June, when tadpoles were free swimming and had resorbed their yolk sacs (stage 25), I counted the surviving tadpoles of all families and divided this number by the number of fertilized eggs as determined on 27 May to obtain a measure of early survival rates, which I will refer to as ''hatchling survival.'' On the same day, I haphazardly selected seven tadpoles per family that I weighed to the nearest milligram and 20 tadpoles that I transferred to outdoor tanks for rearing. A single cross (Se 1 × El 11, see Appendix) did not produce a sufficient number of free-swimming tadpoles for outdoor rearing. All remaining tadpoles were kept in the laboratory to document the occurrence of developmental abnormalities. These tadpoles were discarded after one month.

Rearing Tadpoles

I used 80 plastic tanks (70 cm long × 40 cm wide × 30 cm high) as artificial ponds to raise tadpoles. Holes were drilled in the sides of the tanks to maintain a constant water level even during rainfall, and lids made of shading cloth prevented colonization by invertebrate predators. I arranged the tanks in five spatial blocks to control for any environmental variation within a fenced field at the university. The tanks were filled on 25 May with 80 L of tap water and inoculated with 3.5 g of rabbit chow and 1.5 L of concentrated phyto- and zooplankton on three occasions to start primary production. Each tank was stocked with 20 tadpoles from a single cross. Every block received one replicate cross from each of the 16 population combinations, and crosses were randomly assigned to positions within blocks. Starting with day 5 of the rearing period, I counted the live tadpoles in the tanks every fifth day to obtain a survivorship curve for all crosses. Every 10th day, I temporarily removed a haphazardly selected subsample of seven tadpoles from each tank and weighed them to the nearest milligram. Based on the number of live tadpoles and their mean weight obtained from these censuses, I added supplementary food at a rate of 5% of the mean tadpole weight in a given tank per day and tadpole. I used finely ground *Spirulina* tablets as food and fed the tadpoles every fifth day during the early phase of the rearing period and every second or third day during later development to avoid water pollution due to the increasing amounts of food. The supplementary feeding served two purposes: (1) to provide favorable growth conditions because I wanted to assess tadpole viability and performance under benign environmental conditions; and (2) to eliminate or at least mitigate the effects of density on tadpole growth that I expected under differential mortality.

I defined metamorphosis as the emergence of at least one forelimb (stage 42; Gosner 1960). When tadpoles began to metamorphose, I checked the tanks daily and transferred metamorphs to the laboratory, where they were held in plastic containers at 20°C. When tail resorption was complete (stage 46), metamorphs were weighed to the nearest milligram, euthanized by immersion in MS-222, and frozen at −80°C for later protein electrophoresis. The experiment was ended after 110 days, on 24 September, when only four surviving tadpoles had not yet metamorphosed.

Hemiclone Determination

I used cellulose acetate electrophoresis following standard procedures described in Hebert and Beaton (1993) to genetically characterize the source populations and to determine the different hemiclones within *R. esculenta*. Initially, all animals used for crossings and one offspring from each cross that had produced metamorphs were screened for variation at nine enzymes representing 14 putative loci known to be often polymorphic in waterfrogs. These enzymes included aspartate amino transferase (Aat, EC 2.6.1.1), aconitate hydratase (Aco, EC 4.2.1.3), glucose-6-phosphate isomerase (Gpi, EC 5.3.1.9), glyceraldehyde-3-phosphate dehydrogenase (Gapdh, EC 1.2.1.12), isocitrate dehydrogenase (Idh, EC 1.1.1.42), lactate dehydrogenase (Ldh, EC 1.1.1.27), mannose-6-phosphate isomerase (Mpi, EC 5.3.1.8), phosphoglucomutase (Pgm, EC 5.4.2.2), and phosphogluconate dehydrogenase (Pgdh, EC 1.1.1.44). By comparing the electrophoretic phenotypes of parents and offspring it was possible to assign different alleles at a given locus in *R. esculenta* to either its R or L genome. This analysis revealed that six loci were polymorphic in *R. ridibunda*, that is, either in the Ba individuals or in the R genomes of *R. esculenta* used for crossings: *sAat, Gpi, Ldh-B, Mpi, Pgm-2,* and *Pgdh* (locus designations follow Hotz et al. 1997). Consequently, population samples from the three source populations of *R. esculenta* were only analyzed for variation at these six loci. I defined a hemiclone as a distinct haploid multilocus genotype of an R genome in *R. esculenta*. Obviously, haplotype resolution is limited. R genomes sharing the same multilocus genotype may differ at loci not assayed or they may possess amino acid substitutions that do not result in detectable differences in electrophoretic mobility. Thus, an allozymic hemiclone may represent several hemiclones that happen to share the same allozyme phenotype.

Statistical Analysis

Fertilization rate and hatchling survival were analyzed with mixed-model nested analyses of variance (ANOVAs) using PROC GLM (SAS Institute 1989), testing for the effects of female source, male source, and their interaction, as well as for the effects of individual females and males within sources.

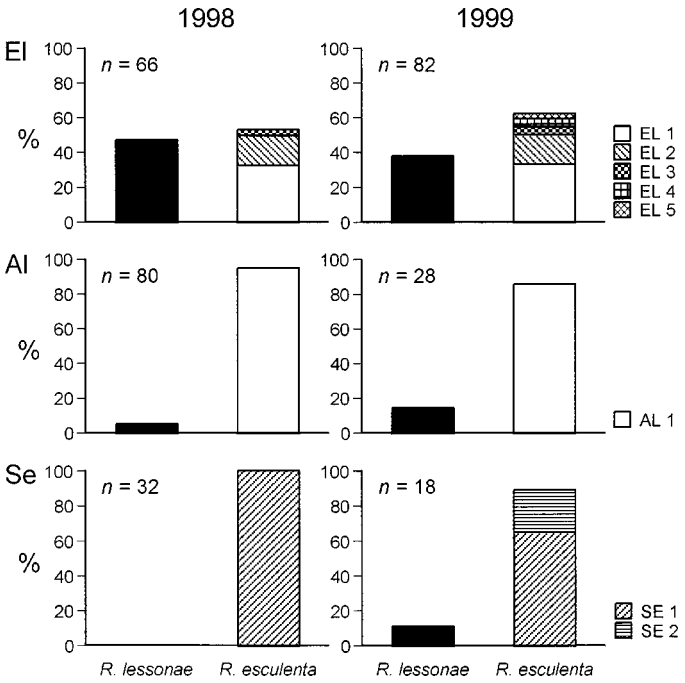

FIG. 3. Composition in percent of the three source populations containing a native *Rana lessonae*/*R. esculenta* mixed population over two years. The percentage of *R. esculenta* is further subdivided into the different hemiclones that could be identified. El, Elliker Auen; Al, Alpnach; Se, Seseglio. *n,* total number of individuals captured in a given year.

Source populations were considered fixed effects, but individuals were randomly chosen from the source populations and therefore considered random effects. The variation among source populations in both sexes was further partitioned into the variation between the *R. ridibunda* source (Ba) and the *R. esculenta* sources (El, Al, and Se), and the variation among the three *R. esculenta* source populations using orthogonal linear contrasts. A RANDOM statement was used to generate mixed-model expected mean squares and error estimates for *F*-tests using Satterthwaite's approximations (SAS Institute 1989). The crossing design was not completely balanced because one female from Ba produced overripe eggs that could not be fertilized and had to be excluded from all analyses. I therefore used Type III sums of squares. Fertilization rate and hatchling survival were measured as proportions and arcsine-square-root transformed before analysis to meet the assumptions of ANOVA (Zar 1999).

The success of crosses was determined by percent survival to metamorphosis and by three larval life-history traits that are reliably associated with fitness in anurans (Berven and Gill 1983; Smith 1987; Berven 1990; R. Altwegg, pers. comm.): size at metamorphosis (mass in milligram after tail resorption, stage 46), larval period (days from the start of the rearing period on 6 June to forelimb emergence, stage 42), and daily growth rate. For size and larval period I used mean values per tank as the unit of analysis because measurements from individuals within tanks were not independent. Growth rates for each cross were calculated from the mean weights of the initial subsamples taken at the start of the rearing period and the mean weights obtained from the regular measurements taken every 10 days thereafter from a subsample of seven tadpoles per cross. Inspection of the growth curves revealed that tadpoles followed an exponential growth trajectory for the first 40 days, after which growth slowed down due to the onset of metamorphosis. I therefore assumed an exponential growth model and calculated the proportional increase in weight per day separately for each of the first four 10-day intervals. These values were subsequently averaged to obtain a single measurement of the daily growth rate per cross. Survival was analyzed with the same statistical model as used for fertilization rate and hatchling survival, except for the inclusion of block as an additional factor to account for potential effects of environmental variation within the tank array. The proportion of survivors to metamorphosis was also transformed by the arcsine of the square root before analysis. Size at metmorphosis, larval period, and growth rate were analyzed with mixed-model nested analyses of covariance (ANCOVAs) containing the same factors as the model for survival plus final density (i.e., the number of survivors until metamorphosis) as a covariate to account for potential density effects that might have arisen despite the supplementary feeding. This is only valid if the final density reflects the density experienced during most of the larval period, that is, if mortality occurred early in development. This was the case in the present study (see Results). For the ANCOVAs of larval life-history traits I used Type IV sums of squares because, as expected, crosses from certain combinations of parental source populations were completely inviable, leading to empty cells in the design.

TABLE 1. Hemiclones defined by the haploid multilocus genotypes of the R genome in *Rana esculenta.* Loci are named as in Hotz et al. (1997); allele designations follow Hotz (1983) and Beerli (1994).

Hemiclone	Diagnostic loci					
	sAat	*Gpi*	*Ldh-B*	*Mpi*	*Pgm-2*	*Pgdh*
AL 1 and EL 1	*e*	*a*	*c*	*c*	*d*	*d*
EL 2	*e*	*a*	*c*	*a*	*d*	*d*
EL 3	*e*	*a*	*a*	*c*	*d*	*d*
EL 4	*e*	*d*	*c*	*c*	*d*	*d*
EL 5	*e*	*a*	*a*	*a*	*d*	*d*
SE 1	*e*	*a*	*c*	*c*	*c*	*e*
SE 2	*e*	*a*	*c*	*c*	*c*	*d*

RESULTS

Composition of Source Populations

In 1998, El was found to contain 47% *R. lessonae* and 53% *R. esculenta.* These figures remained similar in 1999, when I captured 38% *R. lessonae* and 62% *R. esculenta* (Fig. 3). Among *R. esculenta,* three different hemiclones could be distinguished in 1998 ($n = 34$, *R. esculenta* analyzed electrophoretically). In the larger 1999 sample ($n = 52$), I could identify two additional rare hemiclones (Table 1, Fig. 3). Of the 10 *R. esculenta* used for crossings in 1998, two females and three males belonged to hemiclone EL 1, two females and two males to hemiclone EL 2, and one female to hemiclone EL 3. Population Al contained only 5% *R. lessonae* and 95% *R. esculenta* in 1998 and 14% *R. lessonae* and 86% *R. esculenta* in 1999. In both years, all *R. esculenta* analyzed

TABLE 2. Mixed-model nested ANOVA tables for fertilization rate (proportion of eggs fertilized) and hatchling survival (proportion of fertilized eggs developing to tadpole stage 25). Proportions were transformed by the arcsine of the square root before analysis. Satterthwaite approximations were used to generate error estimates for *F*-tests.

Source of variation	Fertilization rate				Survival to stage 25			
	df	MS	*F*	*P*	df	MS	*F*	*P*
Female source	3	0.391	2.023	0.169	3	0.341	3.883	0.045
Rana esculenta vs. *R. ridibunda*	1	0.998	5.144	0.044	1	0.659	7.488	0.021
Among *R. esculenta*	2	0.067	0.345	0.714	2	0.060	0.679	0.529
Male source	3	0.133	0.968	0.440	3	0.007	1.272	0.328
R. esculenta vs. *R. ridibunda*	1	0.340	2.443	0.144	1	0.001	0.174	0.684
Among *R. esculenta*	2	0.030	0.212	0.812	2	0.009	1.814	0.205
Female source × male source	9	0.012	0.815	0.607	9	0.003	0.833	0.591
Female (source)	11	0.194	13.326	<0.001	10	0.088	22.288	<0.001
Male (source)	12	0.139	9.585	<0.001	12	0.005	1.327	0.255
Residual	30	0.015			30	0.004		

belonged to the same hemiclone AL 1 ($n = 71$). Within the resolution provided by the six loci I examined, AL 1 was identical to the most common hemiclone EL 1 in El (Table 1), and may thus represent the same clonal lineage. In Se, only *R. esculenta* was captured in 1998 (Fig. 3). All animals examined electrophoretically ($n = 15$) belonged to the same hemiclone SE 1, which differed at two loci from all hemiclones found on the northern side of the Alps (Table 1). The pattern changed slightly in 1999, when a few (11%) *R. lessonae* were captured and a second hemiclone was identified among the *R. esculenta* examined ($n = 13$). This hemiclone (SE 2) was also different from all hemiclones found in Al and El (Table 1, Fig. 3).

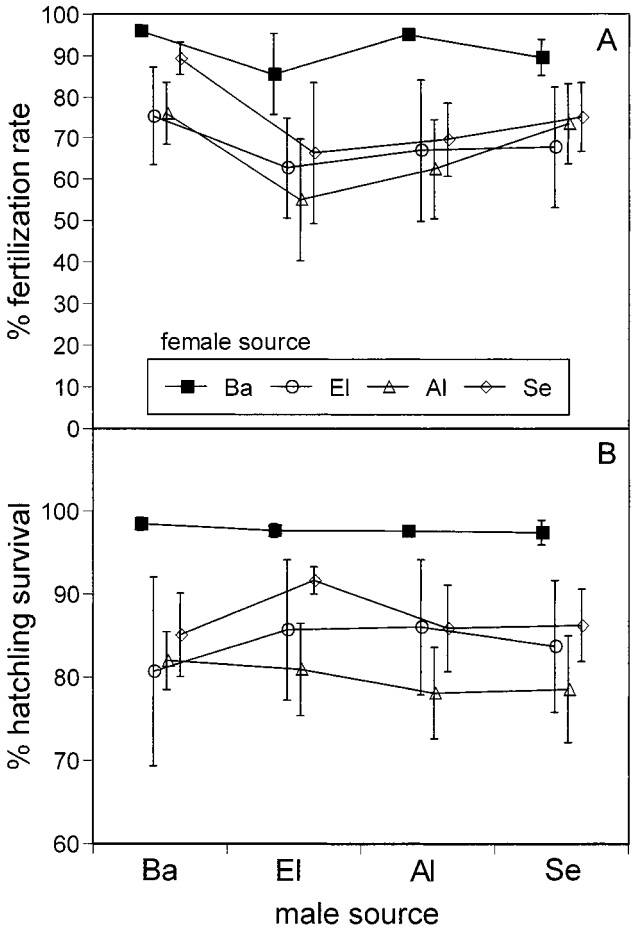

FIG. 4. Interaction of source of females and source of males on fertilization rate and hatchling success (proportion of fertilized eggs developing to tadpole stage 25) of experimental crosses. Values are means and error bars represent ±1 SE. Sources are defined in Figure 3.

Fertilization Rates and Hatchling Survival

Fertilization rates were mainly determined by individual variation among male and female frogs used for crossings (Table 2). Overall, sperm of male *R. ridibunda* from Ba obtained the highest fertilization rates. Similarly, eggs of females from Ba were slightly better fertilized than eggs of females from the other source populations (Fig. 4A). But the overall effect of source was not statistically significant for either sex, nor was there a significant interaction among sources (Table 2). In females, however, the linear contrast between Ba and the other sources indicated that *R. ridibunda* females from that one population produced eggs that were better fertilized than eggs from the three *R. esculenta* populations (Table 2).

Hatchling survival was not significantly affected by the source of males, nor was there significant variation among individual males within sources (Table 2). The source population of females, in contrast, had a significant effect on hatchling survival, and there was large variation among individual females within sources (Table 2). Orthogonal contrasts indicated that hatchling survival of offspring produced by *R. ridibunda* mothers from Ba was higher than hatchling survival of offspring from *R. esculenta* mothers, but there was no significant difference among the three *R. esculenta* sources (Fig. 4B, Table 2). One female from El was declared an outlier and excluded from this analysis because mean hatchling survival of her offspring was less than one fifth of that of any other female.

Survival to Metamorphosis

The source population of females and males, as well as their interaction, had a strong effect on tadpole survival (Table 3). The highly significant linear contrasts between the *R. ridibunda* source and the three *R. esculenta* sources in both

TABLE 3. Mixed-model nested ANOVA table for survival rate (proportion of tadpoles surviving to metamorphosis) of experimental crosses raised in outdoor tanks. Proportions were transformed by the arcsine of the square root before analysis. Satterthwaite approximations were used to generate error estimates for *F*-tests.

Source of variation	df	MS	*F*	*P*
Block	4	0.041	0.270	0.895
Female source	3	1.676	14.241	0.001
Rana esculenta vs. *R. ridibunda*	1	3.731	31.092	<0.001
Among *R. esculenta*	2	0.159	1.324	0.305
Male source	3	0.745	5.484	0.017
R. esculenta vs. *R. ridibunda*	1	1.957	14.326	0.003
Among *R. esculenta*	2	0.148	1.083	0.370
Female source × male source	9	1.341	8.832	<0.001
Female (source)	11	0.120	0.793	0.646
Male (source)	12	0.137	0.901	0.557
Residual	28	0.152		

sexes reflects the fact that all crosses with at least one *R. ridibunda* parent from Ba produced viable tadpoles that successfully completed metamorphosis, whereas many *R. esculenta* × *R. esculenta* crosses completely failed at an early larval stage. There was no significant difference among the *R. esculenta* sources in either sex (Table 3), indicating that offspring from none of the three sources were inherently more or less viable. Instead, survival of tadpoles was mainly determined by the combination of parents they were produced by. Figure 5 depicts the survivorship curves of all crosses grouped by population combination.

Rana esculenta × *Rana esculenta* crossings

Within-population crossings with *R. esculenta* from Al and Se (belonging to only one hemiclone each) produced inviable offspring. Interestingly, the two types of tadpoles suffered from different developmental abnormalities: Al × Al (female × male) tadpoles all had kinky tails and died within the first four weeks of the rearing period (Figs. 5F, 6B). Se × Se tadpoles had swollen, balloonlike bellies (Fig. 6C), a syndrome previously described as oedema by Ogielska (1994). These tadpoles died within the first two weeks of the rearing period (Fig. 5K). Crossings between these two populations (i.e., Al × Se and Se × Al) produced tadpoles with a normal appearance (Fig. 6A), most of which survived and successfully completed metamorphosis (Figs. 5G, J). The five within-population crosses produced by *R. esculenta* from El (belonging to three different hemiclones) were not homogeneous. Three crosses were viable and two crosses failed completely (Fig. 5A). El × Se and Se × El crosses all produced metamorphs, but survival was highly variable and in some cases very low (Figs. 5C, I). Between-population crossings with parents from El and Al (i.e., Al × El and El × Al) only produced three viable families, the remaining seven crosses failed to survive until metamorphosis (Figs. 5B, E). Remarkably, the same three individual *R. esculenta* from El that produced viable offspring in crossings with *R. esculenta* from Al were also involved in the three El × El crossings that produced viable tadpoles (see Appendix).

Viability of crosses within and between populations Al and Se could be predicted from the combination of parental hemiclones: Within-hemiclone crosses were inviable, between-hemiclone crosses were viable. Similarly, crosses between populations Se and El, also separated by the Alps and therefore not sharing any hemiclones, all produced metamorphs, although survival was much more variable. Viable within-population crosses were only obtained from El parents that belonged to three different hemiclones, and fully inviable between-population crosses were only obtained between populations El and Al, which had one allozymic hemiclone in common (Table 1). In these cases, however, offspring viability could not be predicted from hemiclone combinations: two crosses from parents belonging to the same allozymic hemiclone produced metamorphs, and five crosses from parents belonging to different hemiclones failed completely (Fig. 7).

Crossings with Rana ridibunda

Tadpoles from *R. ridibunda* × *R. ridibunda* crosses showed high survival with no more than one of 20 tadpoles per cross dying before successful metamorphosis (Fig. 5P). Tadpoles possessing one clonal and one sexual R genome from crossings between *R. esculenta* and *R. ridibunda* were generally equally viable. However, six of these crosses showed a spell of high mortality early in development, after which the remaining tadpoles survived well and completed metamorphosis (Figs. 5D, H, L–N). A striking feature of these crosses was that roughly half of the tadpoles failed to grow, producing an increasingly bimodal size distribution until tadpoles of the smaller size class died.

Larval Life-History Traits

There was no significant effect of final density on any of the larval life-history traits measured, suggesting that the supplementary feeding efficiently mitigated density effects resulting from differential mortality (Table 4). Block effects were not significant for mass at metamorphosis and larval period, but marginally significant for growth rate (Table 4). Mass at metamorphosis was not significantly affected by any of the factors in the ANCOVA. However, there was significant variation among source populations of females for both the time to metamorphosis and daily growth rate. Orthogonal contrasts revealed that this variation was mainly due to *R. ridibunda* females from Ba producing tadpoles with shorter larval periods and higher growth rates than *R. esculenta* from El, Al, and Se, among which variation was not significant (Table 4, Fig. 8). Variation among females within sources

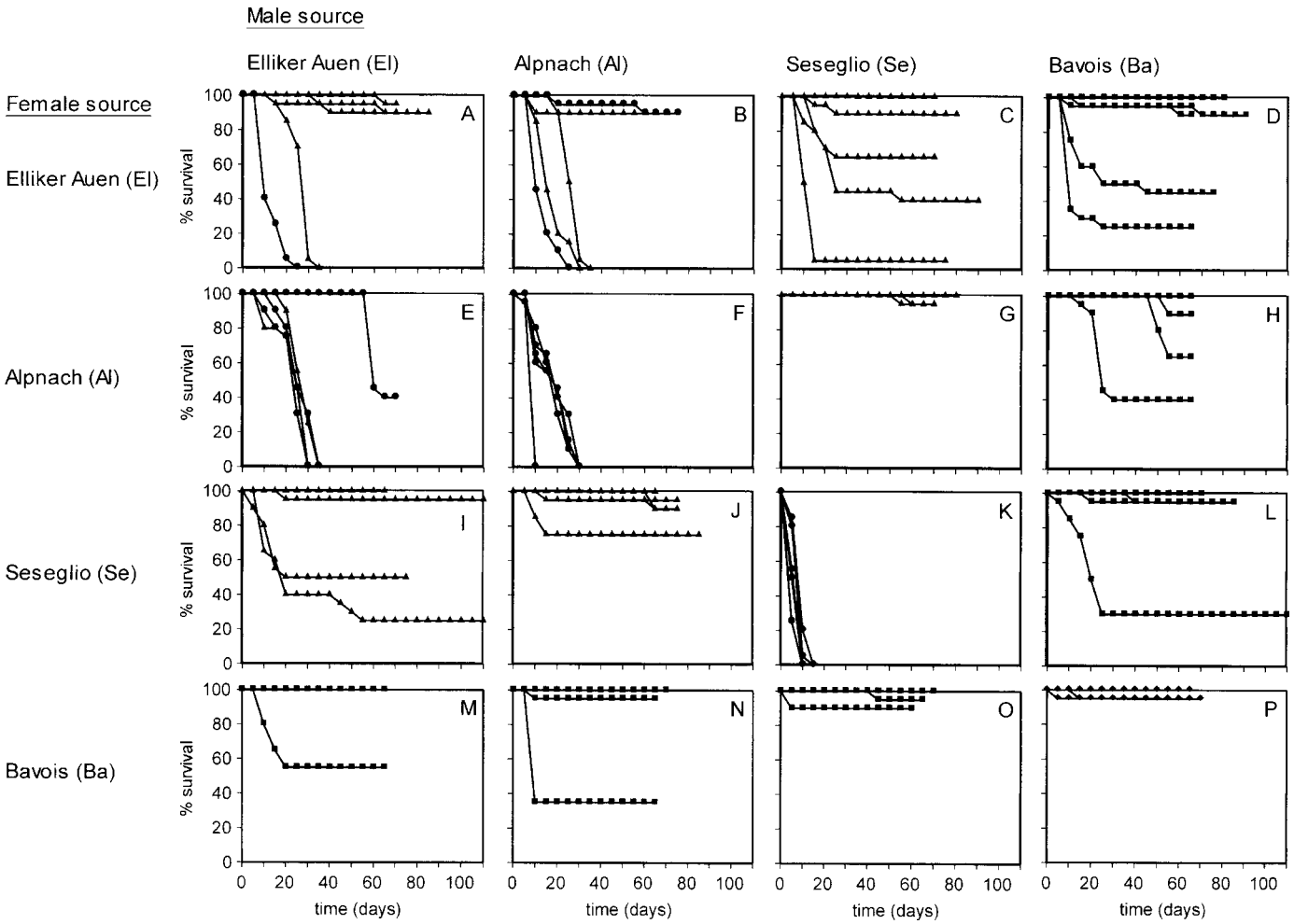

FIG. 5. Survivorship curves of all experimental crosses grouped by the source populations of parents. Parents from Elliker Auen, Alpnach, and Seseglio were *Rana esculenta* transmitting clonal R genomes; parents from Bavois were *R. ridibunda* transmitting sexual R genomes in their gametes. Each cross was raised in a separate tank at an initial density of 20 tadpoles per tank. Curves end when the last tadpole either reached metamorphosis or died. Circles represent crosses possessing two clonal genomes of the same allozymic hemiclone, triangles represent crosses with two clonal genomes of different hemiclones, squares represent crosses with one clonal and one sexual genome, and diamonds represent crosses with two sexual genomes. All $n = 5$ crosses per population combination, except for Figures 6I, M, N, O, and P, where $n = 4$.

was also marginally significant for both of these traits (Table 4). Neither larval period nor growth rate were significantly affected by male source; there was significant variation among males within sources for growth rate, however, but not for larval period (Table 4).

DISCUSSION

The finding that self-incompatible hemiclones can produce viable *R. ridibunda* offspring when combined with different hemiclones provides evidence for the hypothesis that the inviability of offspring from natural matings between two *R. esculenta* is caused by fixed deleterious mutations on their R genomes. This is consistent with the operation of Muller's ratchet, which has been shown by simulation to cause the rapid fixation of deleterious mutations in haploid clonal populations (Higgs and Woodcock 1995; Charlesworth and Charlesworth 1997). However, the present data do not allow a distinction between mutations that occurred after a hemiclone was formed (i.e., Muller's ratchet) and mutations that were segregating in the original sexual *R. ridibunda* population and became frozen in a clonal R genome through hybridization. Both may contribute to the low fitness of intrahemiclonal crosses. Yet, two characteristics of *R. esculenta* make the operation of Muller's ratchet very likely. First, the immigration of hybrids from areas of sympatry of the parental species into central Europe probably included many subsequent colonization events, each representing a founder event with only a limited number of individuals. Founder events promote genetic drift and thereby the fixation of spontaneous deleterious mutations (Chao 1990; Clarke et al. 1993). Second, recessive deleterious mutations in clonal R genomes are sheltered from selection by the sexual L genome in hybrids, again making their fixation more likely (Nei 1970). Furthermore, in populations with a low proportion of the sexual host *R. lessonae* (e.g., Al and Se in this study), hybrids may undergo repeated bottlenecks due to the limited availability of suitable mates.

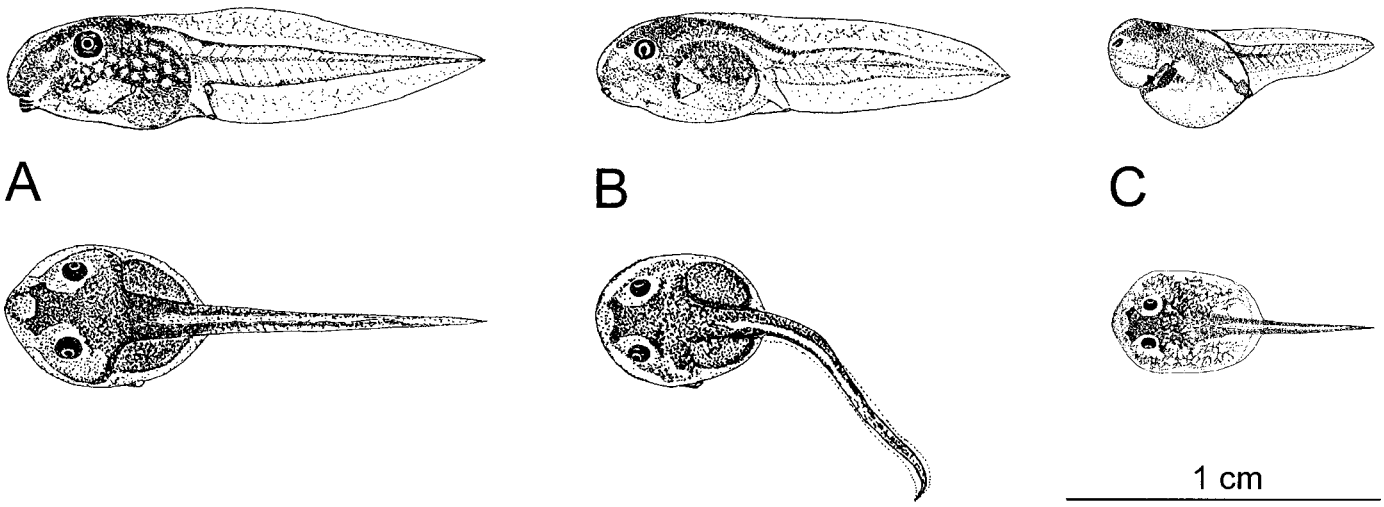

FIG. 6. Lateral and dorsal view of representative tadpoles from *Rana esculenta* × *R. esculenta* crosses between populations Al and Se (A), within population Al (B), and within population Se (C). Tadpoles raised in the laboratory for closer examination were photographed on 17 June 1998, approximately three weeks after hatching. Drawings were produced from these photographs.

To determine the relative importance of mutations acquired at and after hemiclone formation, respectively, a quantitative estimate of the average mutational load per genome in a sexual *R. ridibunda* population is required. So far, no such estimate is available for any frog species, but the possibility to artificially produce new hemiclonal lineages by crossing the two parental species provides a powerful way to obtain such an estimate for *R. ridibunda*. This project is currently pursued by H. Hotz and collaborators and should yield the necessary information to distinguish between the alternatives (H. Hotz, pers. comm.).

The most straightforward result of this study comes from the crosses between populations Al and Se. As expected, *R. esculenta* produced inviable offspring when crossed with other hybrids from their own population, whereas the same individuals produced fully viable offspring when crossed with *R. esculenta* from the other population. Al × Al tadpoles suffered from different developmental abnormalities than Se × Se tadpoles, indicating that the responsible mutations arose at different loci in hemiclones AL 1 and SE 1. Crossings involving *R. esculenta* from El produced more ambiguous results. Some within-population crosses successfully completed metamorphosis, but many between-population crosses with *R. esculenta* from Al failed. This may reflect the fact that hybrids in El consist of several hemiclones, one of which may be identical to the single hemiclone in Al and therefore share the same deleterious alleles. That one or several deleterious alleles exist in both populations is supported by the fact that only individuals associated with successful El × El crossings produced viable offspring when crossed to *R. esculenta* from Al. But this evidence is weak due to the low number of crosses available for comparison.

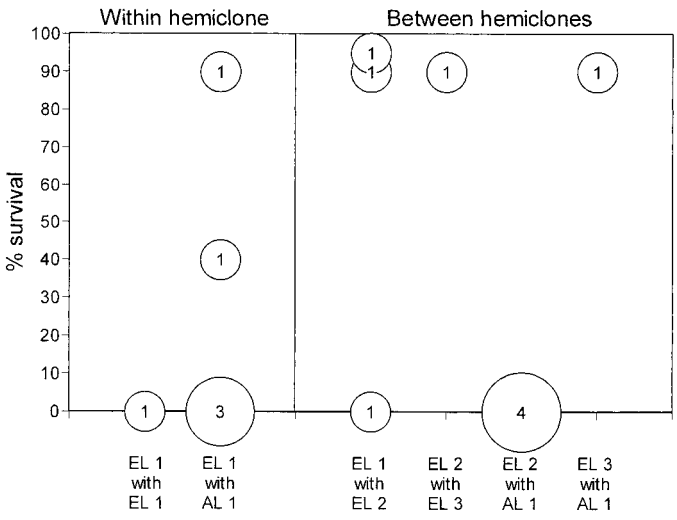

FIG. 7. Percent survival of all *Rana esculenta* × *R. esculenta* crosses within population El and between populations El and Al, grouped by hemiclone combination. Numbers inside bubbles indicate the number of crosses with equal survival rates. Note that EL 1 and AL 1 share the same haploid multilocus genotype and therefore represent the same allozymic hemiclone.

While a hemiclone present in El may exist in Al, too, it is unlikely to also exist in Se, because Se is separated from the other populations by the Alps. Hybrids there probably stem from different areas of sympatry between the parental species and must have immigrated via different routes. Accordingly, no allozymic hemiclone was found on both sides of the Alps. Still, some crossings between populations El and Se produced very few survivors, suggesting that, at least in some cases, two clonal R genomes not sharing a common ancestry may be poorly compatible. It cannot yet be answered whether this is because they happen to carry deleterious alleles at the same loci or because of synergistic interactions among mutations at different loci.

A detailed analysis of crossings within population El and between populations El and Al revealed that in these cases, tadpole viability could not be predicted from the combination of allozymic hemiclones. One obvious problem here is that the resolution of hemiclone determination with allozymes is

TABLE 4. Mixed-model nested ANCOVA tables for mass at metamorphosis, time to metamorphosis (days), and daily growth rates of *Rana ridibunda* tadpoles. Mean values per cross were taken as the unit of analysis. Satterthwaite approximations were used to generate error estimates for *F*-tests.

Source of variation	df	Mass at metamorphosis			Time to metamorphosis			Daily growth rate		
		MS	*F*	*P*	MS	*F*	*P*	MS[1]	*F*	*P*
Final density	1	6852.167	0.084	0.777	3.100	0.276	0.611	0.046	1.314	0.278
Block	4	19808.139	0.244	0.907	24.615	2.191	0.143	0.123	3.484	0.050
Female source	3	48408.408	0.847	0.485	225.555	9.455	0.001	0.958	11.838	<0.001
R. esculenta vs. *R. ridibunda*	1	75346.218	1.938	0.191	428.516	12.834	0.004	2.990	25.776	<0.001
Among *R. esculenta*	2	51155.399	1.315	0.308	79.261	2.371	0.139	0.136	1.177	0.344
Male source	3	102127.882	1.657	0.206	9.225	0.499	0.688	0.119	1.361	0.293
R. esculenta vs. *R. ridibunda*	1	9210.118	0.171	0.687	35.268	1.651	0.223	0.180	1.672	0.220
Among *R. esculenta*	2	138236.808	2.566	0.118	14.743	0.690	0.520	0.103	0.961	0.410
Female source × male source	7	103954.704	1.279	0.350	19.065	1.697	0.216	0.095	2.677	0.077
Female (source)	11	38889.656	0.478	0.879	33.432	2.976	0.048	0.116	3.274	0.036
Male (source)	12	53865.202	0.663	0.753	21.367	1.902	0.158	0.108	3.046	0.044
Residual	10	81275.060			11.233			0.035		

[1] Values represent MS × 1000.

limited. In a study on clonal variability among 30 unisexual strains of the hybridogenetic fish *Poeciliopsis monacha-lucida*, Angus and Schultz (1979) could identify 18 distinct histocompatibility genotypes using the more sensitive technique of tissue grafting. An electrophoretic survey with a resolution comparable to the present study had only identified eight allozymic hemiclones among the same 30 strains (Vrijenhoek et al. 1978). Thus, it is possible that some crossings between two *R. esculenta* of the same allozymic hemiclone actually combined R genomes belonging to different clonal lineages that happen to share the same multilocus genotype. Indeed, Hotz et al. (2001) have shown that allozymic hemiclones can be further subdivided by using more variable microsatellite markers. This may explain why some crosses within hemiclones were viable, but not why some crosses between distinguishable hemiclones were inviable (Fig. 7), unless we assume that allelic changes in the diagnostic loci arose after clone formation, for which there is presently no evidence such as new or silent allozyme alleles (Graf and Polls Pelaz 1989).

This result can be explained, however, if the different hemiclones coexisting in El do not represent evolutionarily independent lineages. This may come about if hybrid × hybrid matings occasionally produce viable *R. ridibunda* offspring that reach sexual maturity. Such *R. ridibunda* cannot found independently reproducing populations, because due to the fact that clonal R genomes generally contain an X chromosome, they are all females (Berger et al. 1988). But these females are expected to exhibit normal Mendelian meiosis and thus to recombine the two clonal genomes they inherited. Renewed hybridizations with *R. lessonae* could then found new hemiclones, some of which may be purged from deleterious mutations (Schmidt 1993) or have remaining mutations linked to new combinations of marker alleles, making it impossible to predict the viability of hybrid × hybrid offspring based on the combination of allozymic hemiclones. This explanation was also suggested by G.-D. Guex, H. Hotz, and R. D. Semlitsch (unpubl. ms.) to explain the inviability of crosses between two coexisting hemiclones in another Swiss population.

So far, there is one well-documented case of the formation of fertile female *R. ridibunda* from hybrid × hybrid matings in a L-E-system (Hotz et al. 1992), but the viable within-population crosses of this experiment suggest that the same is likely to occur in El. Indeed, recent field captures in El show that in 1999, large numbers of all-female *R. ridibunda* metamorphs were produced by pairs of *R. esculenta*, some of which survived for at least one year (Vorburger 2001b). The possibility for episodic recombination of this kind appears to be conditional on the initial presence of more than one hemiclone in a population, which is the case in many L-E systems examined so far (e.g., Uzzell and Berger 1975; Hotz 1983; Semlitsch et al. 1997). Hemiclonal diversity can be maintained in a population if hemiclones are ecologically distinct (frozen niche variation model; Vrijenhoek 1979), which is supported by empirical evidence in *R. esculenta* (Semlitsch et al. 1997). So, clearly, future studies have to critically consider the possibility of derived rather than ancient hemiclones occurring in L-E systems with several coexisting hemiclones (G.-D. Guex, H. Hotz, and R. D. Semlitsch, unpubl. ms.).

All crossings between *R. esculenta* and *R. ridibunda* parents produced viable progeny, confirming the results of previous experiments (e.g., Berger 1967; Kawamura and Nishioka 1986). This contrasts with hybridogenetic fish of the genus *Poeciliopsis*, where substantial incompatibilities have accumulated between clonally and sexually inherited genomes (Leslie and Vrijenhoek 1980). This difference may reflect the presumed younger age of some clonal R genomes compared to clonal *monacha* genomes in *Poeciliopsis*, where the oldest known clonal lineage has an estimated age of at least 100,000 generations (Quattro et al. 1992). Although there appeared to be no general reduction in survival of crosses with one *R. esculenta* and one *R. ridibunda* parent, in six of these crosses roughly half of the tadpoles failed to grow and died within the first four weeks of the rearing period (Fig. 5). The reason for this mortality is yet unknown and requires further investigation.

Considering the larval life-history traits of survivors, tadpoles with *R. ridibunda* mothers performed better in that they grew faster and reached metamorphosis earlier than tadpoles with *R. esculenta* mothers. In fathers, however, there was no

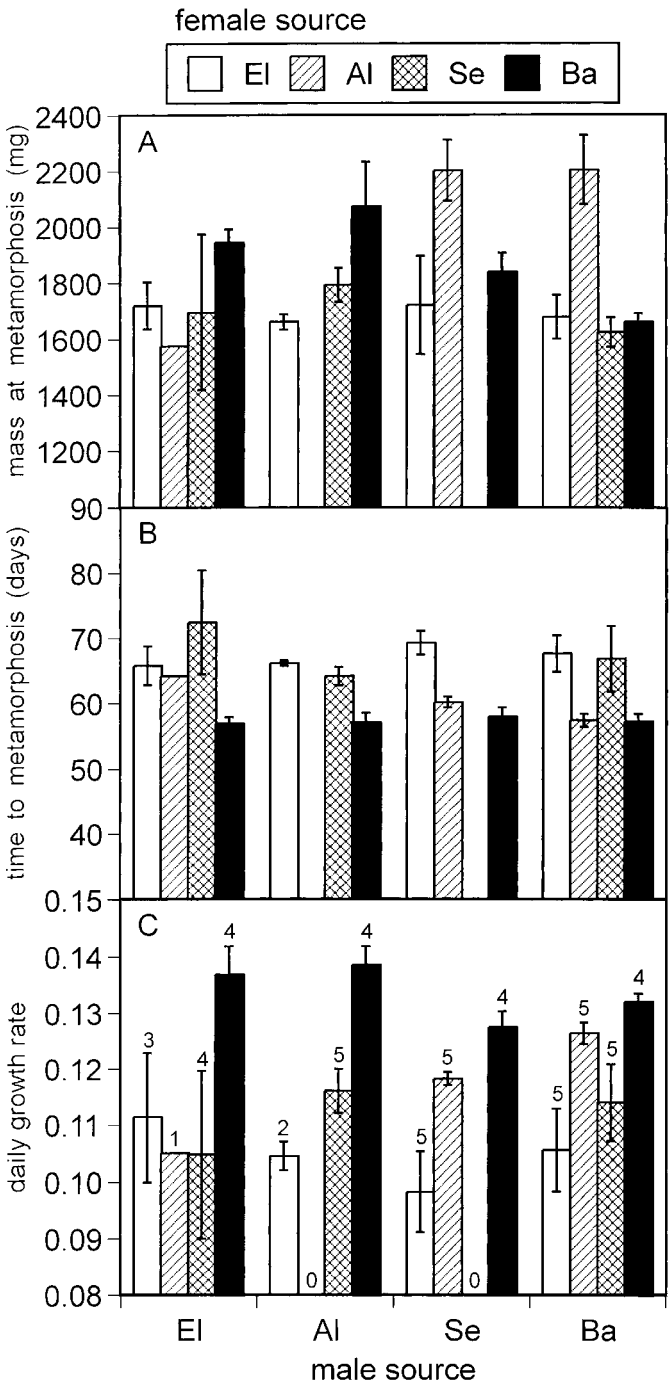

FIG. 8. Mean mass at metamorphosis (A), mean larval period (B), and daily growth rate (C) of tadpoles as a function of the source population of female and male parents. Error bars depict ±1 SE, numbers above error bars in Figure 8C indicate the number of crosses represented by each bar for all three responses.

significant difference between taxa for any of the larval life-history traits measured, indicating that maternal effects are responsible for the reduced performance of offspring from *R. esculenta* females. Because clonal R genomes do contain a substantial load of deleterious mutations, this result suggests that their heterozygous effects on fitness are weak, at least too weak to be detected in this experiment. This appears surprising at first, because deleterious mutations have been shown not to be completely recessive (Simmons and Crow 1977; García-Dorado and Caballero 2000). However, there is ample evidence that the effects of deleterious mutations depend on environmental conditions (Kondrashov and Houle 1994). For example, mutation accumulation due to relaxed selection in *Drosophila* led to a rapid decline in fitness when fitness was assayed under competitive conditions, but not when assayed under benign conditions (Shabalina et al. 1997). Similarly, stressful conditions enhance the negative effects of inbreeding (e.g., Wolfe 1993; Jimenez et al. 1994; Meagher et al. 2000). Thus, although heterozygous fitness effects of clonal R genomes were weak under the benign conditions of this study, they may become significant in a more stressful environment. However, in a separate experiment addressing this question specifically, I detected no negative effects of clonal genomes in the heterozygous state even under stressful conditions (Vorburger 2001a). It seems worth considering that if the inviability of within-hemiclone crosses is mainly the result of mutations frozen at hemiclone formation, clonal R genomes may not have a much higher mutational load than an average genome in a sexual *R. ridibunda* population.

Offspring of *R. ridibunda* females from Ba not only benefitted from maternal effects in terms of growth rate and larval period, fertilization and survival of fertilized eggs to tadole stage 25 was also enhanced. Again, there were no comparable differences between taxa in males. Together, these findings indicate a reduced egg quality in *R. esculenta*. But this conclusion is tentative because *R. ridibunda* and *R. esculenta* came from only one and three populations, respectively. If real, the reduced egg quality of *R. esculenta* is likely to be a direct consequence of problems in oogenesis due to its hybrid state rather than the result of genetic deterioration, or else we would expect similar effects in hybrid males. This is supported by the finding that field-caught *R. esculenta* suffer from the same abnormalities in gametogenesis as F_1 hybrids produced in the laboratory, although the R genomes of the latter have not been transmitted clonally for generations (Wagner and Ogielska 1993; Ogielska and Bartmanska 1999).

Conclusions

Due to its hemiclonal mode of reproduction and the fact that it occurs in both sexes (unlike other hybridogenetic vertebrates), the hybrid frog *R. esculenta* provides the unique opportunity to combine different clonal genomes in the same individual to study their effects on fitness. Taking advantage of this property, I found support for the hypothesis that different lineages of clonally transmitted R genomes are fixed for different recessive deleterious mutations, explaining the commonly observed inviability of offspring from hybrid × hybrid matings in natural populations. Such mutations may have preexisted in the original sexual *R. ridibunda* population and became frozen in clonal R genomes through hybridization or they may have occurred after hemiclone formation and became fixed through the advance of Muller's ratchet. Additional experiments are required to assess the relative importance of these two processes. Although the homozygous effects of these mutations are dramatic, their heterozygous

effects on fitness appear to be weak. This may in part explain the high ecological success of *R. esculenta* (Semlitsch 1993), which benefits from heterosis due to its hybrid condition (Hotz et al. 1999), but does not appear to suffer greatly from genetic deterioration of its clonally inherited R genome.

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Appendix

Summary of fertilization rate, hatchling survival, survival to metamorphosis, mean mass at metamorphosis, mean larval period, and daily growth rate for all experimental crosses. The first column gives the parents (female × male) of each cross. Parents are labeled with a two-letter code for their population of origin and a number identifying the individual. El, Elliker Auen; Al, Alpnach; Se, Seseglio (*Rana esculenta* sources); and Ba, Bavois (*R. ridibunda* source). The second and third column indicate the allozymic hemiclone as defined in Table 1 of *R. esculenta* females and males, respectively. SD for mass at metamorphosis and larval period are given in parentheses.

Cross	Hemiclone of female	Hemiclone of male	% Fertilization	% Hatchling survival	% Survival to metamorphosis	Mass at metamorphosis (mg)	Larval period (days)	Daily growth rate
El 1 × El 13	EL 2	EL 1	62.7	60.6	90	1706 (190)	64.11 (4.21)	0.118
El 4 × El 18	EL 1	EL 2	38.6	90.9	95	1868 (106)	61.58 (1.71)	0.127
El 7 × El 11	EL 1	EL 1	39.7	10.0	0	—	—	—
El 8 × El 14	EL 2	EL 1	53.4	95.7	0	—	—	—
El 9 × El 17	EL 3	EL 2	95.9	95.4	90	1582 (144)	71.50 (4.51)	0.089
El 1 × Al 14	EL 2	AL 1	59.3	61.7	0	—	—	—
El 4 × Al 16	EL 1	AL 1	21.6	92.5	90	1634 (226)	66.56 (2.01)	0.102
El 7 × Al 12	EL 1	AL 1	65.6	5.9	0	—	—	—
El 8 × Al 18	EL 2	AL 1	97.1	95.1	0	—	—	—
El 9 × Al 11	EL 3	AL 1	89.7	94.6	90	1688 (134)	65.72 (2.30)	0.107
El 1 × Se 15	EL 2	SE 1	59.4	60.2	40	1951 (241)	74.63 (7.95)	0.087
El 4 × Se 11	EL 1	SE 1	30.0	88.1	90	1789 (166)	70.06 (3.62)	0.102
El 7 × Se 12	EL 1	SE 1	50.3	11.9	65	2120 (123)	64.08 (2.02)	0.120
El 8 × Se 13	EL 2	SE 1	94.3	93.1	100	1642 (199)	66.70 (1.66)	0.103
El 9 × Se 14	EL 3	SE 1	87.2	93.3	5	1100 (—)	71.00 (—)	0.079
El 1 × Ba 13	EL 2	—	53.2	48.2	25	1372 (134)	63.40 (1.14)	0.121
El 4 × Ba 20	EL 1	—	56.1	81.9	90	1744 (212)	75.50 (6.68)	0.089
El 7 × Ba 16	EL 1	—	67.7	12.5	90	1808 (196)	59.44 (4.18)	0.125
El 8 × Ba 18	EL 2	—	95.4	96.2	100	1732 (284)	69.25 (4.18)	0.097

APPENDIX. CONTINUED

Cross	Hemiclone of female	Hemiclone of male	% Fertilization	% Hatchling survival	% Survival to metamorphosis	Mass at metamorphosis (mg)	Larval period (days)	Daily growth rate
El 9 × Ba 15	EL 3	—	96.4	96.3	45	1739 (290)	70.56 (4.07)	0.096
Al 1 × El 11	AL 1	EL 1	37.8	89.1	0	—	—	—
Al 3 × El 13	AL 1	EL 1	84.1	87.0	40	1574 (253)	64.13 (1.36)	0.105
Al 4 × El 14	AL 1	EL 1	14.3	80.6	0	—	—	—
Al 6 × El 18	AL 1	EL 2	92.8	88.2	0	—	—	—
Al 9 × El 17	AL 1	EL 2	45.5	59.6	0	—	—	—
Al 1 × Al 12	AL 1	AL 1	78.1	80.9	0	—	—	—
Al 3 × Al 14	AL 1	AL 1	92.7	86.7	0	—	—	—
Al 4 × Al 18	AL 1	AL 1	72.5	84.7	0	—	—	—
Al 6 × Al 16	AL 1	AL 1	39.1	81.4	0	—	—	—
Al 9 × Al 11	AL 1	AL 1	29.5	56.6	0	—	—	—
Al 1 × Se 12	AL 1	SE 1	84.8	79.4	100	2562 (169)	62.37 (1.86)	0.121
Al 3 × Se 15	AL 1	SE 1	83.9	87.7	100	2281 (147)	59.95 (3.57)	0.117
Al 4 × Se 13	AL 1	SE 1	78.8	89.0	95	1900 (185)	62.74 (1.82)	0.116
Al 6 × Se 11	AL 1	SE 1	84.8	82.5	100	2094 (121)	59.20 (1.58)	0.116
Al 9 × Se 14	AL 1	SE 1	34.6	54.0	95	2167 (158)	58.79 (2.46)	0.121
Al 1 × Ba 16	AL 1	—	70.4	80.8	89.5[1]	2067 (236)	55.77 (3.33)	0.132
Al 3 × Ba 13	AL 1	—	88.0	83.0	40	2610 (183)	54.75 (4.40)	0.129
Al 4 × Ba 18	AL 1	—	76.0	86.4	100	1860 (181)	60.35 (2.85)	0.124
Al 6 × Ba 20	AL 1	—	94.6	89.9	65	2178 (531)	57.92 (4.72)	0.121
Al 9 × Ba 15	AL 1	—	50.7	69.6	100	2301 (291)	58.20 (3.74)	0.126
Se 1 × El 11	SE 1	EL 1	5.5	88.9	—[2]	—	—	—
Se 2 × El 14	SE 1	EL 1	50.8	90.8	95	1875 (98)	69.72 (3.85)	0.112
Se 6 × El 18	SE 1	EL 2	92.4	94.8	100	1710 (121)	58.40 (2.41)	0.121
Se 8 × El 13	SE 1	EL 1	92.7	96.0	50	2257 (329)	66.20 (3.26)	0.125
Se 10 × El 17	SE 1	EL 2	90.1	87.5	25	937 (110)	95.33 (12.01)	0.061
Se 1 × Al 12	SE 1	AL 1	56.5	65.6	90	1934 (188)	61.39 (4.06)	0.120
Se 2 × Al 18	SE 1	AL 1	94.6	93.6	95	1711 (181)	63.79 (4.69)	0.121
Se 6 × Al 16	SE 1	AL 1	57.9	91.6	100	1931 (133)	61.75 (2.15)	0.113
Se 8 × Al 14	SE 1	AL 1	51.3	86.4	90	1652 (155)	64.44 (2.33)	0.124
Se 10 × Al 11	SE 1	AL 1	87.4	92.0	75	1725 (76)	69.27 (4.96)	0.102
Se 1 × Se 12	SE 1	SE 1	81.2	72.5	0	—	—	—
Se 2 × Se 13	SE 1	SE 1	91.1	88.1	0	—	—	—
Se 6 × Se 11	SE 1	SE 1	88.2	94.9	0	—	—	—
Se 8 × Se 15	SE 1	SE 1	44.9	95.1	0	—	—	—
Se 10 × Se 14	SE 1	SE 1	69.7	80.3	0	—	—	—
Se 1 × Ba 16	SE 1	—	75.2	66.0	100	1592 (181)	58.20 (3.30)	0.118
Se 2 × Ba 18	SE 1	—	91.7	90.0	95	1453 (180)	65.63 (7.08)	0.125
Se 6 × Ba 20	SE 1	—	97.6	94.2	100	1613 (154)	59.75 (4.18)	0.114
Se 8 × Ba 13	SE 1	—	88.2	84.4	95	1707 (164)	64.47 (4.85)	0.125
Se 10 × Ba 15	SE 1	—	93.9	90.6	30	1760 (373)	86.17 (15.14)	0.088
Ba 1 × El 13	—	EL 1	99.0	96.8	100	1995 (198)	55.60 (3.59)	0.136
Ba 4 × El 14	—	EL 1	57.4	96.5	100	1999 (165)	55.20 (4.64)	0.151
Ba 6 × El 11	—	EL 1	86.6	97.2	55	1975 (333)	59.18 (4.09)	0.131
Ba 10 × El 17	—	EL 2	98.9	100.0	100	1804 (166)	57.70 (3.70)	0.129
Ba 1 × Al 14	—	AL 1	96.7	97.5	95	1939 (233)	55.95 (3.73)	0.142
Ba 4 × Al 18	—	AL 1	92.4	96.6	100	2232 (279)	57.75 (3.31)	0.143
Ba 6 × Al 12	—	AL 1	95.4	98.5	35	2420 (496)	54.00 (5.29)	0.140
Ba 10 × Al 11	—	AL 1	95.4	97.6	100	1700 (174)	60.70 (4.23)	0.129
Ba 1 × Se 15	—	SE 1	78.2	93.0	95	1867 (194)	57.47 (2.84)	0.122
Ba 4 × Se 13	—	SE 1	98.8	99.4	100	1668 (242)	60.95 (4.49)	0.125
Ba 6 × Se 12	—	SE 1	92.5	98.1	90	1989 (234)	54.22 (2.90)	0.135
Ba 10 × Se 14	—	SE 1	88.5	99.0	100	1834 (182)	59.10 (2.47)	0.128
Ba 1 × Ba 13	—	—	93.8	96.1	100	1576 (296)	54.80 (4.92)	0.132
Ba 4 × Ba 18	—	—	93.3	99.6	95	1683 (231)	57.74 (5.15)	0.136
Ba 6 × Ba 16	—	—	97.2	99.0	95	1658 (209)	56.32 (4.85)	0.129
Ba 10 × Ba 15	—	—	99.3	99.0	100	1726 (177)	60.10 (3.78)	0.131

[1] Nineteen instead of 20 tadpoles correspond to 100% because one tadpole was accidentally killed.
[2] No value because not enough tadpoles were available for outdoor rearing.