

The importance of heterozygosity in a frog's life

Dirk S. Schmeller · Julia Schregel · Michael Veith

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Abstract High genetic variability may increase metabolic efficiency and thus allows responding to environmental challenges as limits to adaptation are approached. Therefore, it has been suggested that high genetic variability contributes strongly to the fitness of an individual. Survival to high age may thus depend on high genetic variability, and genetically variable individuals may have a higher survival rate to high ages in comparison to less variable sympatric conspecifics. Such a heterozygosity \times age relationship might be more readily detectable in stressful as compared to benign environments. For testing the relationship between age and heterozygosity, we genetically analyzed 71 individuals of the frog species *Rana perezi* from a total of seven populations at 13 allozyme loci. The age of the individuals was determined by skeletochronology. We found effects on age of both environment and allozyme heterozygosity, especially in populations with

high stress regimes. A significant heterozygosity \times age relationship has so far rarely been shown in natural populations. The result of our analysis suggests that more heterozygous individuals have a higher longevity and may be an important source of genetic variability of a population, likely contributing to a stabilization of the effective population size.

Keywords Anura · Amphibia · Longevity · Heterozygosity \times fitness relationship · Storage effect · Population stability

Introduction

In many species, heterozygous individuals show increased fitness in comparison to homozygous conspecifics. Fitness has been measured among other traits by developmental stability, growth rate, survival, or reproductive success. For example, individuals with higher heterozygosity are more developmentally stable in the rare plant species *Gentiana pneumonanthe* L. (Oostermeijer et al. 1995); in the common frog *Rana temporaria*, larval survival is related to genetic variability, especially under stressful environments (Lesbarreres et al. 2005); in the bivalve *Spisula ovalis*, growth is positively related to heterozygosity, probably because of inbreeding effects (David et al. 1997); in *Colia* butterflies, phosphoglucose isomerase polymorphism is correlated with flight ability and male mating success (Watt et al. 1985; Watt 1983). Absence of a positive relationship between fitness and heterozygosity has also been observed numerous times, however (e.g., Booth et al. 1990; Savolainen and Hedrick 1995; Whitlock 1993). The generally weak association of fitness and heterozygosity

D. S. Schmeller (✉) · J. Schregel · M. Veith
Department of Ecology, Institute of Zoology,
Johannes Gutenberg-University Mainz,
Saarstraße 21,
55099 Mainz, Germany
e-mail: ds@die-schmellers.de

M. Veith
IBED—Institute for Biodiversity and Ecosystem Dynamics,
University of Amsterdam,
1098 SM Amsterdam, The Netherlands

Present address:
D. S. Schmeller
Department of Conservation Biology,
UFZ—Helmholtz Centre for Environmental Research,
Permoserstrasse 15,
04318 Leipzig, Germany

(Britten 1996) has been explained by the marker system and the number of markers used, the traits investigated, and the environment chosen (Balloux et al. 2004; Cassel et al. 2001; Wang et al. 2002). The latter especially may be crucial to detecting genetic variability \times fitness correlations, as such an association can be hidden when conditions are optimal, but appear in stressful environments (e.g., Cassel et al. 2001).

For explanation of genetic variability \times fitness associations, three alternative hypotheses have been put forward (Hansson and Westerberg 2002). Lerner (1954) suggested that the superiority of heterozygotes results from a better buffering capacity in different environments. This direct-effect hypothesis attributes heterozygote advantage to functional overdominance. Functional overdominance occurs when heterozygous individuals have an intrinsically higher fitness compared to homozygotes because of a higher efficiency of their metabolism (Zink et al. 1985), or different catalytical properties of enzymes compared to homozygotes (Hansson and Westerberg 2002; Mitton 1997). Hence, selection directly acts on the loci analyzed. The local effect hypothesis proposes that linkage disequilibrium causes genetic variability \times fitness associations between neutral markers and fitness loci in their chromosomal vicinity. Under the general effect hypothesis, genetic variability \times fitness associations result from effects of homozygosity at all loci distributed across the genome.

In general, life-history traits, such as survival and longevity, have been found to be excellent candidates for detecting genetic variability \times fitness correlations (Coltman and Slate 2003; Lesbarreres et al. 2005). Life-history evolution is driven by various abiotic and biotic environmental factors and has been subject to various studies (e.g., Hairston and Walton 1986; Laurila et al. 1997; Vrijenhoek and Pfeiler 1997; Altwegg 2002). In amphibians, links between tadpole and adult fitness are strong: large size at metamorphosis is strongly associated with survival and fecundity of adults (e.g., Guarino et al. 1998; Miaud et al. 1999) because performance during later life is influenced by carryover effects (Räsänen et al. 2003) from conditions experienced during early development (e.g., Goater 1994). Hence, age can be used as a surrogate of fitness in amphibians.

In this study, we ask whether longevity of the frog species *Rana perezi* is dependent on increased levels of allozyme heterozygosity, especially in stressful environments. We hypothesize that environmental stress enhances the expression of recessive deleterious alleles (Hedrick 1986; Hedrick and Kalinowski 2000), increasing genetic variability \times fitness correlations in stressful as compared to benign environments. We assume that variation in allozyme polymorphism reflects variation in genome-wide heterozygosity, following the direct-effect hypothesis (Hansson and Westerberg 2002). We tested these predictions by using

data from populations in environmentally variable localities in two areas.

Materials and methods

The species investigated, *R. perezi*, belongs to the western Palearctic water frog group. The species spends its almost entire activity period in or close to the water, occupying marshes, small ponds, water channels, and shallow waters of different sizes (Schmeller, personal observation). We collected *R. perezi* ($N=71$) from four populations (8, 13, 20, and 24) at the Rhône delta (Camargue) and from three populations (1–3) along the Mediterranean coastline of southern France during one breeding period (Table 1). The mean distance between populations of the Camargue area was estimated to be 8.2 km, while the average distance between Camarguean populations and the populations along the Mediterranean coastline was estimated to be 172.9 km. The Camargue is a wetland area of about 820 km² with a strong selective regime. The selective pressures comprise (1) high water dynamics (Golterman 1995; El-Habr and Golterman 1987), increasing the risk of desiccation for *R. perezi* (e.g., Rowe and Dunson 1995); (2) predation risk due to numerous bird and fish species in high

Table 1 Age, as determined by skeletochronology, sample size (N), and observed heterozygosity by age group (H_O) in different populations (Pop) of *R. perezi* together with population means for salinity (μ S), relative saturation of dissolved oxygen (O_{2rel}), and water acidity (pH)

Pop	Age	N	H_O	μ S	O_{2rel}	pH	Coordinates
1 ^a	1	7	0.033	926	130	6.8	42°34.39N
	2	2	0.038				03°00.48E
2 ^a	1	6	0.064	642	90	6.9	42°39.14N
	2	1	0.000				02°54.39E
3 ^a	1	5	0.062	1,856	110	5.6	43°00.47N
	2	12	0.096				03°02.56E
	3	1	0.000				
8	1	3	0.026	1,150	160	5.3	43°36.43N
	2	4	0.096				04°20.34E
	3	1	0.077				
13	1	15	0.031	1,050	200	5	43°28.71N
	2	3	0.077				04°38.99E
20	1	2	0.153	450	30	5.3	43°30.32N
	2	1	0.000				04°40.02E
	3	2	0.231				
24	1	4	0.096	1,020	110	6.3	43°27.99N
	2	2	0.077				04°52.86E
Σ		71					

GPS coordinates are given for each population. Population numbers marked with superscript “a” are non-Camargue populations. Populations are numbered from south to northeast.

abundances (e.g., Kayser et al. 2003; Poizat et al. 2004); and (3) strong human impact on the water system made evident by drainage schemes, dikes, rice paddies, and salt pans (Pierce and Crivelli 1994).

Especially not only salinity and heavy metal concentration, but also the water acidity and the oxygen availability have various impacts on different life stages of amphibians (Grillitsch and Chovanec 1995; Viertel 1999). We therefore measured acidity (pH), salinity (μS), and the relative oxygen saturation ($O_{2\text{rel}}$) of water in each population (Table 1) during the sampling period, which spanned 3 months during the summer period. The measurements were taken in the approximate center of each habitat using a multiparameter measuring instrument of Hanna Instruments. To avoid some of the known obstacles in receiving data on these environmental variables, we took three measurements per population, one each by midday, afternoon, and during the night. We took measures at approximately the same time of day at each habitat over the whole sampling season (for data, see Appendix 1). The mean acidity of all populations of the Camargue was 5.5, while in the other populations, acidity was lower (mean $\text{pH}=6.4$; $t_5=1.97$, $P=0.105$). The relative oxygen level was slightly higher in populations located in the Camargue (124%) as compared to the more southern populations (110%; $t_5=0.31$, $P=0.766$), which holds true also for salinity (Camargue=918 μS , other=1141 μS ; $t_5=0.62$, $P=0.561$).

The age of *R. perezii* was determined using skeletochronology because age of temperate zone amphibian species can easily be inferred from analyses of bone cross sections. The method relies on visible lines of arrested growth (LAGs) in bone cross sections, which are built during times of a lower metabolism during hibernations or estivations (Castanet et al. 1977; Hemelaar and Gelder 1980). In *R. perezii*, LAGs are usually very distinct, allowing for a good inference of age (Patón et al. 1991). One finger was used for skeletochronological age determination. It was decalcified, dehydrated, and embedded in Historesin® (LEITZ, Wetzlar, Germany). After embedding, fingers were cut into 11 μm sections with a rotation microscope. From each finger, every tenth section (about 40 in total) was transferred to a microscopic slide. We usually analyzed cross sections from the middle between the phalangeal diaphyses. This procedure guaranteed ideal cross sections with distinct LAGs. The microscopic slide was moistened with slightly soaped water and placed on a heating plate, which raised the temperature of the water to about 45°C to stretch the sections. The dried sections were stained with Cresyl violet to visualize the LAGs and were subsequently covered with glass. The LAGs of several sections of the same specimen were counted under the microscope at a magnification of 200× or 400×.

For determination of individual heterozygosity, we screened 39 enzyme systems, of which 13 loci were polymorphic and consistently scorable using standard procedures of allozyme electrophoresis applied on muscle and liver tissue samples (Hebert and Beaton 1993; Table 2). The individual observed heterozygosity (H_o) and allele frequencies were calculated using the program Genetix v4.05 (Belkhir et al. 1996–2004). H_o represents the proportion of heterozygous loci of an individual. For species identification, we followed the procedure reported earlier using diagnostic alleles at several loci (Schmeller et al. 2005a).

Table 2 Allozyme loci used to determine individual heterozygosities and allele frequencies over the whole sample

Enzyme	Tissue	Buffer	EC number	Allele	Frequency
<i>ogdh</i>	Liver	TG 8.5	1.1.1.8	1	0.0070
				2	0.9930
<i>6pgdh</i>	Muscle	TB 8.9	1.1.1.44	2	0.0282
				3	0.9225
				4	0.0423
				5	0.0070
<i>aat</i>	Liver	TC 8.2	2.6.1.1	2	0.3429
				3	0.6571
<i>ahh</i>	Liver	TG 8.5	3.3.1.1	1	0.0845
				2	0.9155
<i>ck</i>	Muscle	TG 8.5	2.7.3.2	1	0.9930
				2	0.0070
<i>fh</i>	Liver	TG 8.5	4.2.1.2	1	0.0141
				2	0.9648
				3	0.0141
				5	0.0070
<i>gapdh</i>	Muscle	TC 8.2	1.2.1.12	2	0.0775
				3	0.9014
				4	0.0141
				5	0.0070
<i>ldh</i>	Liver	TG 8.5	1.1.1.27	2	0.0141
				4	0.9859
<i>me</i>	Muscle	TG 8.9	1.1.1.40	1	0.9859
				3	0.0141
				3	0.0141
<i>mpi</i>	Liver	TM 7.0	5.3.1.8	1	0.0141
				2	0.9718
				3	0.0141
<i>mpr</i>	Muscle	TB 8.9	–	1	0.9789
				2	0.0141
				3	0.0070
<i>gpi</i>	Liver	TG 8.5	5.3.1.9	1	0.0141
				3	0.9859
<i>pgm</i>	Muscle	TM 7.0	5.4.2.2	1	0.0070
				2	0.9930

We used standard procedures of cellulose acetate allozyme electrophoresis (Hebert and Beaton 1993) with several buffer systems (TG Tris–glycine, TB Tris–borate, TC Tris–citrate, TM Tris–maleate, PP phosphate; pH is given as decimal number).

The statistical analysis

The partial intercorrelation of the environmental parameters O_{2rel} , pH, and μS ($R_{19} \text{ pH} \times \mu S = -0.297$, $P = 0.191$; $R_{19} \text{ pH} \times O_{2rel} = -0.322$, $P = 0.155$; $R_{19} \mu S \times O_{2rel} = 0.530$, $P = 0.014$) made it necessary to run a principal component analysis (PCA) to retrieve environmental components from these three parameters. The first axis (component I, eigenvalue=1.59) explained 53% of the total variance, with the pH (loading=0.475) negatively related to O_{2rel} (-0.626) and μS (-0.619). The second axis (component II, eigenvalue=0.83) explained 28% of the total variance (loadings: pH=-0.879; $O_{2rel} = -0.308$; $\mu S = -0.363$). Component III (eigenvalue=0.58), explained 19% of the variance (loadings: pH=-0.036; $O_{2rel} = -0.716$; $\mu S = 0.697$).

In a first step, we explored effects of environmental parameters and heterozygosity on the longevity (age) using *t* tests and one-factor ANOVAs. In a second step, we used generalized linear models (GENMOD, SAS Institute, Cary, NC, USA; 1999), including population and area effects on the individual heterozygosity in the model (link function=log). The dependent variable age was Poisson-distributed. We started the GENMOD analysis with a fully saturated model, dropping nonsignificant factors step by step. The goodness-of-fit assessment revealed an overdispersion of the data, making it necessary to use a scaling procedure to avoid an underestimation of standard errors. The scale parameter was estimated by the square root of deviance divided by the degrees of freedom.

Results

In our sample, the observed heterozygosity ranged from 0 to 0.308 with an average of 0.066 ± 0.073 , equivalent to approximately one polymorphic locus per individual. The most numerous age class was the first age class with 42 individuals (59% of total sample), followed by the class of 2-year olds with 25 individuals (35%). The maximum age of *R. perezii* was 3 years (four individuals, 6%). The average age was 1.46 ± 0.605 years (Table 1).

Over all samples, the heterozygosity level of individuals in their first year (0.051) was significantly lower than in older individuals (0.088; $t_{69} = 2.11$; $P = 0.038$). The difference remains when habitat type is added as a factor (ANOVA: $F_{3,67} = 2.74$; $P = 0.050$; Fig. 1). The heterozygosity difference between these two age groups of Camargue as compared to non-Camargue populations is not significant (ANOVA: $F_{3,67} = 1.72$; $P = 0.170$; Fig. 2). Comparing

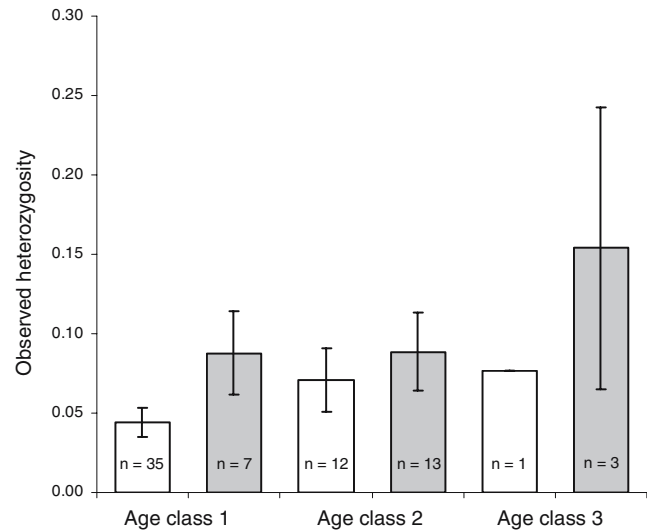


Fig. 1 Observed heterozygosity in the different age classes with regard to the habitat types B (white) and C (gray). Habitat type B is defined by intermediate flood influence and oxygen concentrations, while C habitats cover peat bogs, which are more remote to freshwater sources. The error bars indicate the standard error, and sample sizes are given by *n*

the 1-year olds with the older individuals within each region reveals a significant difference between the age groups in the Camargue (1 year=0.052; 2 and 2+ years=0.101; $t_{35} = 1.94$; $P = 0.030$; Fig. 2) but not in the non-Camargue populations (1 year=0.052; 2 and 2+ years=

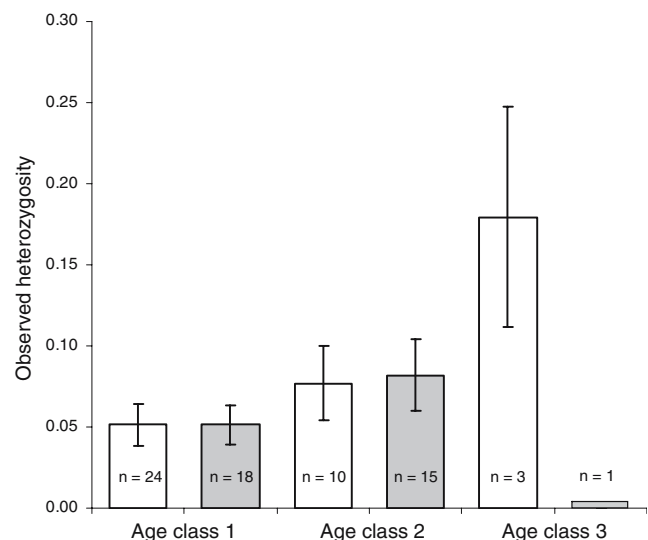


Fig. 2 Observed heterozygosity in the different age classes with regard to the region Camargue (white) and non-Camargue (gray). The two regions differ in their stress regimes, considering predator pressure, risk of desiccation, water chemical factors, and human impact. The error bars indicate the standard error, and sample sizes are given by *n*

0.077; $t_{32}=1.08$; $P=0.289$; Fig. 2). The heterozygosity difference between the 1-year olds and the older individuals is not supported considering population as factor (ANOVA: $F_{13,57}=1.41$; $P=0.183$).

The more complex model, considering environmental components and including area as a factor, revealed a significant effect of heterozygosity ($\chi^2_{2,65}=7.93$, $P=0.019$) and component III ($\chi^2_{1,67}=15.37$, $P<0.001$) on the longevity. Replacing the area by a population effect supported a significant effect of heterozygosity ($\chi^2_{7,62}=20.59$, $P=0.004$) and the environmental component III ($\chi^2_{1,62}=9.20$, $P=0.002$) but not of the environmental components I ($\chi^2_{1,62}=0.09$, $P=0.761$) and II ($\chi^2_{1,62}=0.56$, $P=0.456$). The relationship between heterozygosity and age is driven by populations 8 ($\chi^2_1=6.81$, $P=0.009$) and 20 ($\chi^2_1=6.00$, $P=0.014$).

Discussion

The most salient finding of our study is the influence of both heterozygosity and environment on the longevity of the frog species *R. perezi*. The relationship is driven by populations in the Camargue region, characterized by a more stressful environment as compared to non-Camargue populations. Our findings thus support the hypothesis that heterozygosity \times fitness correlations are most obvious under stressful conditions (Cassel et al. 2001).

In particular, the more stressful environments in the Camargue created by human impact, high water dynamics, and a high abundance of predators, especially in birds (Kayser et al. 2003) and fishes (Poizat et al. 2004), made the relationship between age and heterozygosity more obvious. Furthermore, the high water dynamics in breeding sites (Pierce and Crivelli 1994) increases the risk of desiccation (Rowe and Dunson 1995). Desiccation is likely in species of water frogs, as specimen of that species group spent most of their active life in or close to the water body of their breeding site. Their ability to prevent desiccation is weak, leading to rather certain death even of adult individuals, if water bodies dry out during summertime (Schmeller and Jakob, personal observation).

Generally, the age of *R. perezi* in our study was low (maximum observed age: 3 years) as compared to the maximum age observed on the Iberian Peninsula (up to 6 years; Esteban et al. 1996). The species colonized France after the last glacial event (Arano et al. 1994). Comparisons of French and Spanish populations (Schmeller 1999) suggest that French populations have lost most of their genetic variability because of founder effects. The low age

of *R. perezi* in France may be linked to its low heterozygosity and a low adaptability to the new and more stressful environmental conditions. Our sample sizes across populations and high age classes are low, reducing the overall power of our analysis. Nevertheless, our comparisons of different age groups in different populations and regions all reveal higher heterozygosity of older individuals as compared to younger ones. The relative increase of heterozygous individuals is obvious in a majority of the populations studied, although not significant, probably because of small sample sizes. We believe, nonetheless, that the general effect of heterozygosity and environment on age is robust.

In our study, we do not address the possible stress provoked by the sympatry of *R. perezi* with other water frogs (e.g., Schmeller et al. 2005b; Pagano et al. 2001a), especially with the hybrid *R. grafi*, which is present in all of the populations included in our study. Few studies have demonstrated that the success of hybrid frogs depends on niche partitioning along an ecological gradient (Pagano et al. 2001b), suggesting that direct competition on resources is reduced. Mating competition must occur, however, because *R. grafi* has to mate with *R. perezi* to maintain its lineage (e.g., Schmeller 2004). This possibly increases stress on *R. perezi* males during the mating period. Our data do not allow the analysis of this factor.

In summary, our results contribute to the understanding of the maintenance of genetic variability and its importance for longevity. Longevity depends on primary selection on stress resistance and metabolic efficiency (Parsons 2002). Our data suggest that survival to higher age is linked to increased levels of heterozygosity, which is assumed to be closely linked to the adaptability to changing and stressful environments (e.g., Wang et al. 2002). Greater longevity and higher lifetime reproductive success of individuals with greater heterozygosity might buffer breeding seasons with low recruitment and help maintain most of the genetic information of a population (storage effect; Warner and Chesson 1985). Such individuals would thus reduce the variance of genetic variability and thereby reduce fluctuations of the effective population size.

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Appendix 1

Environmental measurements for the different populations

Table 3 Environmental measurements for the different populations

Population	μS	$\text{O}_{2\text{rel}}$	pH	Time of day
1	928	133	6.7	Morning
	905	135	6.9	Afternoon
	945	120	6.7	Night
	926	130	6.8	Average
	20.07	8.14	0.12	sd
2	648	90	6.9	Morning
	660	100	7	Afternoon
	619	80	6.7	Night
	642	90	6.9	Average
	21.08	10.00	0.15	sd
3	1,774	110	5.5	Morning
	1,925	120	5.7	Afternoon
	1,868	100	5.6	Night
	1,856	110	5.6	Average
	76.25	10.00	0.10	sd
8	972	160	5.6	Morning
	1,301	190	5.2	Afternoon
	1,178	125	5.2	Night
	1,150	160	5.3	Average
	166.24	32.53	0.23	sd
13	982	190	5	Morning
	1,143	210	5	Afternoon
	1,024	195	4.9	Night
	1,050	200	5	Average
	83.51	10.41	0.06	sd
20	425	40	5.3	Morning
	476	25	5.6	Afternoon
	448	25	5	Night
	450	30	5.3	Average
	25.54	8.66	0.30	sd
24	1,049	105	6.1	Morning
	1,077	115	6.6	Afternoon
	935	110	6.2	Night
	1,020	110	6.3	Average
	75.22	5.00	0.26	sd

μS Salinity, $\text{O}_{2\text{rel}}$ relative oxygen content, pH acidity of the water, sd for standard deviation

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