

## AGE AND SIZE OF HYBRID WATER FROGS: THE ROLE OF GENOTYPE AND ECOLOGY

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**ABSTRACT:** Where fecundity, mortality, immigration, or emigration differ among species, genotypes, environments, sexes, or age and size classes, we must expect differences in the composition and dynamics of populations and communities. However, as shown in this study on all-hybrid populations of the hemiclinal waterfrog *Pelophylax esculentus*, the reverse conclusion is not necessarily true: Population differences in genotype composition were not paralleled by differences in genotype-specific age and size distributions. We investigated 12 ponds with different ratios of diploid (LR) and triploid (LLR and LRR) hybrids and tested whether the pond-to-pond ratio differences result from differential survival of the three genotypes under different ecological conditions (selection hypothesis). This should then be reflected in the age and size distribution of the frogs. Age (determined through skeletochronology) and size (measured as snout-vent length) were related to eight ecological variables that in previous studies had been found to affect genotype ratios: temperature, dissolved oxygen, pH, pond size, amounts of submerged and floating pond vegetation, and proportions of forest and wetland area within 20 m of the pond. Only two of these eight variables had a significant effect on growth and survival: frogs in larger ponds were, on average, older and larger than frogs in smaller ponds, and body size also increased with water temperature. Since these relationships did not differ between genotypes and sexes, we conclude that ecological conditions are unlikely to exert their influence on population structure via differential individual performance. The conclusion is supported by our finding that across the 12 study ponds there was no significant relationship between the proportion of a particular genotype  $\times$  sex category and the average age and size of the individuals belonging to that category. An alternative to the selection hypothesis is offered by the recently proposed gamete pattern hypothesis. It states that pond-to-pond differences in genotype ratios arise from different gamete production patterns of LLR, LR, and LRR males and females. But, so far, support for this hypothesis is also weak.

**Key words:** Age and size distribution; Hybrids; *Pelophylax (Rana) esculentus*; Ploidy; Population structure; Selection; Skeletochronology

THE COMPOSITION and dynamics of populations and communities is determined by four basic processes: Birth and immigration increase the number of individuals, death and emigration decrease it. These processes, in turn, are usually related to the age, size, or quality of the interacting individuals. Depending on their specific interests, scientists study these links from different directions. Evolutionary biologists, for instance, start at the population and community level, and ask how different environments select for different life-history traits of the individuals, such as age and size at maturity, number and size of offspring, or age-specific dispersal, reproductive investment, and mortality (Stearns, 1992). Conversely, population ecologists and population geneticists start from given life-history

traits at the level of individuals and ask how these affect events at the levels of populations and communities. Answers to this latter question can have practical implications in conservation biology, breeding programs, and harvesting from natural populations (Newman, 1993; Gosling and Sutherland, 2000). But they are also of academic interest. If, for instance, we do not find the expected relationship between traits at individual and higher levels, we will have to search for factors other than life-history traits that might be responsible for the structure and dynamics of the investigated population or community, including stochastic effects that are particularly important in small populations (Smith et al., 2006). In this study on Palearctic Water Frogs (*Pelophylax esculentus*, formerly *Rana esculenta*), we investigate whether the composition of populations is related to genotype- and environment-related age and size structure of its members. After not finding such a

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TABLE 1.—Typical offspring genotypes and sexes (f, m) arising from matings between the three major adult genotypes found in the natural all-hybrid populations of *Pelophylax esculentus* in Sweden. Adults with the LRR genotype are mostly females; LRR males are extremely rare. Offspring genotypes are derived from the prevailing gamete types (in italics) produced by males and females of the three genotypes. From these offspring types only the hybrid types (shown in bold) survive to adulthood and reproduce, whereas the parental types (LL, RR) die before metamorphosis. On the basis of crossing experiments by Jakob and Arioli (2007) and Christiansen (2009).

	Males	<b>LLR</b>	<b>LR</b>	<b>LRR</b>
Females	Gametes	<i>L<sub>x,y</sub></i>	<i>R<sub>x</sub></i>	<i>R<sub>x</sub></i>
<b>LLR</b>	<i>L<sub>x</sub></i>	<b>LL<sub>f/m</sub></b>	<b>LR<sub>f</sub></b>	<b>LR<sub>f</sub></b>
<b>LR</b>	<i>LR<sub>x</sub>/R<sub>x</sub></i>	<b>LLR<sub>f/m</sub>/LR<sub>f/m</sub></b>	<b>LRR<sub>f</sub>/RR<sub>f</sub></b>	<b>LRR<sub>f</sub>/RR<sub>f</sub></b>
<b>LRR</b>	<i>R<sub>x</sub></i>	<b>LR<sub>f/m</sub></b>	<b>RR<sub>f</sub></b>	<b>RR<sub>f</sub></b>

relationship, we suggest an alternative explanation and call for further studies.

### Study System

*Pelophylax esculentus* is a hybrid that originally derived from the mating between the Pool Frog *P. lessonae* (genotype LL) and the Lake Frog *P. ridibundus* (genotype RR; Berger, 1968). Although both parental species can live in pure populations, the hybrid usually cannot. This results from a strange reproductive mode, known as “hybridogenesis.” It was first observed by Schultz (1969) in unisexual fishes of the genus *Poeciliopsis* and later described for *P. esculentus* by Berger (1977), Graf and Polls Pelaz (1989), and Günther (1990). Hybridogenetic animals eliminate one of their parental genomes during gametogenesis before meiosis and transmit the remaining genome clonally to eggs or sperm cells. The next hybrid generation is produced by *P. esculentus* mating with the parental species whose genome they have discarded. This forces the hybrid into sympatry with *P. lessonae* (LE system) or *P. ridibundus* (RE system). Despite wide geographical overlap, there is a tendency for LE systems dominating in western and central Europe and for RE systems dominating in the east; in some areas the hybrid lives in mixed populations with both parental species (Günther, 1990; Plötner, 2005).

Although sympatry and mating between *P. esculentus* and one or both parental species constitute the normal and most widespread pattern, there are exceptions. The most puzzling one is that of all-hybrid *P. esculentus* breeding populations consisting of diploid LR and triploid LLR or LRR adults (EE system).

Puzzling is not only the fact that hybrids have become reproductively independent from the parental species, but also how these all-hybrid populations are maintained. Under the usual pattern of gamete production, five of nine mating combinations between hybrids should result in offspring with the parental genotypes LL or RR (Table 1). These do, indeed, occur among tadpoles but disappear until the adult stage, indicating strong selection against the parental species (Chapter 3 in Arioli, 2007).

### Population Composition and Individual Performance

Before our own study, such all-hybrid populations had been described for certain areas in Denmark, Germany, Poland, Slovakia, Sweden, and some other European countries (Borkin et al., 1999; Lada, 1999; Mikulíček and Kotlík, 1999; Rybacki and Fog, 1999), but sample sizes were too small or study periods too short for making quantitative statements about genotype compositions in different ponds and their stability or fluctuation over time. In our long-term field study over 6 yr, on the basis of capture–mark–recapture (CMR) data for more than 5000 adult frogs from 12 ponds in southern Sweden, we could show that pond-specific genotype compositions persisted over the years, and ponds differed in their ecological conditions (details will be provided under Results). We therefore hypothesized that pond-to-pond differences are, at least in part, due to environment-related differences in survival and growth of LLR, LR, and LRR. Additional justification for this “selection hypothesis” comes from various studies that suggest different adaptations of L and R genomes and, hence, different performance of genotypes under different

TABLE 2.—Environmental factors that influence population structure or individual performance of larval and adult *Pelophylax esculentus* in all-hybrid populations (EE system) and mixed populations of hybrids and parental species (LER system). Compiled from different studies.

Environmental factor	System	Effect on	
		Population structure	Individual performance
Increasing size of pond	LER	Shift from LL to RR <sup>1</sup>	
+ decreasing vegetation in pond			
+ increasing wetland area around pond	EE	Shift from LLR to LRR <sup>2</sup>	
Decreasing vegetation in pond	EE	Increasing LRR <sub>F</sub> proportions <sup>3</sup>	
Increasing forest area around pond	EE	Shift from LRR to LLR <sup>3</sup>	
Decreasing oxygen	EE	Decreasing LRR proportions <sup>3</sup>	
	LER	Decreasing RR/LLratio <sup>4</sup>	
Decreasing pH	LER	Decreasing RR/LL ratio <sup>4</sup>	
Decreasing temperature	LER	Larvae: Shift from LL to LR <sup>7</sup>	Decrease in larval growth and survival: RR > LR > LL <sup>5,6</sup> Larvae: LL develop and survive better in higher temperature than LR <sup>7</sup> Adults: LL prefer higher temperature than RR <sup>8</sup> ; freezing tolerance: LL < LR < RR <sup>9</sup>

<sup>1</sup> Hohenweg Peter et al. (2002).

<sup>2</sup> Jakob et al. (2010).

<sup>3</sup> Christiansen and Reyer (2011).

<sup>4</sup> Pagano et al. (2001).

<sup>5</sup> Plenet et al. (2000a).

<sup>6</sup> Plenet et al. (2000b).

<sup>7</sup> Negovetic et al. (2001).

<sup>8</sup> Sinsch (1984).

<sup>9</sup> Voituron et al. (2005).

ecological conditions. In mixed parental–hybrid populations of diploid *P. lessonae* (LL), *P. esculentus* (LR), and *P. ridibundus* (RR), larval performance varies with ecological conditions, and adults show different habitat preferences. In all-hybrid populations the proportions of LLR, LR, and LRR are also, to some extent, related to environmental conditions such as water temperature, oxygen content, pond size, and type of the surrounding area. More details and the relevant references are presented in Table 2.

The selection hypothesis yields two straightforward predictions. (1) Those environmental factors that favor a particular genotype (measured by its relative frequency in the pond) should also improve growth (measured by body size) and survival (measured by age) of that genotype. (2) In each pond, individuals of the dominating genotype should, on average, grow bigger and survive better (i.e., get older) than individuals of the rarer genotypes. In this study, we test these predictions by measuring body size and determining age through skeletochronology and then relating these variables to pond

ecology and genotype composition. Before addressing these primary goals, we investigate in a first step how reliable skeletochronology is for estimating age. In a second step we describe the general age and size patterns for the three genotypes and the two sexes.

## MATERIALS AND METHODS

### Source Populations

We determined the age and snout–vent length (SVL) of 899 *P. esculentus* from 12 ponds in the province of Skåne (southern Sweden; Fig. 1). The frogs used for this study were caught during two breeding seasons (2002, 2003), but catching continued for other purposes from 2004 to 2007. During these 6 yr, we monitored all 12 ponds twice per season, with the first period from between early May and first week of June and the second period between mid-June and mid-July. Catching and recatching days were calculated relative to May 1 (= day 1). We caught more than 5000 adults (> 55 mm), measured their SVLs, marked them

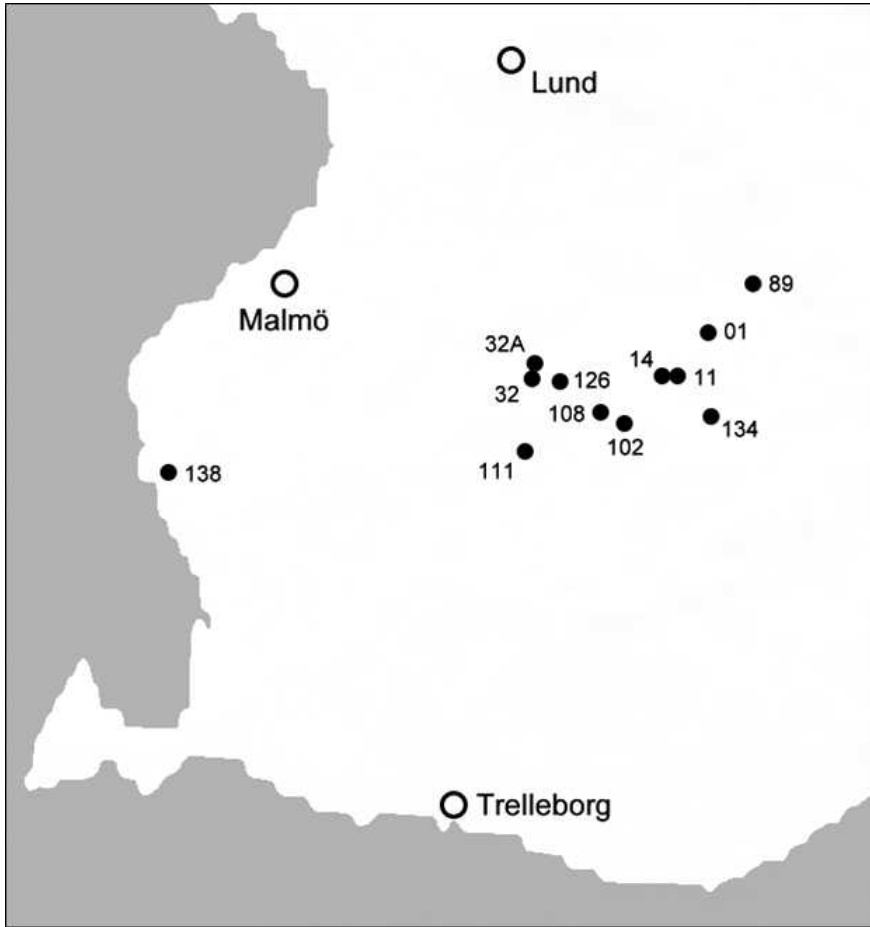


FIG. 1.—Locations of the 12 study ponds (= numbered dots) in the province of Skåne (southern Sweden), relative to three major cities (= circles).

individually with transponders (Trovan ID101 transponders, Pamela AG, Switzerland), and took a toe clip before releasing them back into the pond. The toe clip was later used for determination of age (see below) and genotyping through microsatellite analysis. Since some of the markers we used are genotype specific (L or R) and show genome dosage effects, they allowed unambiguous identification of all occurring frog types. We found six LL and seven LLRR frogs. All others were diploid or triploid hybrids in the following relative frequencies: 42.6% LLR, 53.2% LR, and 4.2% LRR among males; 18.2% LLR, 45% LR, and 36.9% LRR among females. Although there was some annual (but no

seasonal) variation in these genotype proportions, the most pronounced differences existed among ponds. This was especially true for the composition of triploids: In some ponds, they were almost exclusively LLR and in others LRR (Fig. 2).

More details on pond locations, ecological conditions and their recording, catching methods, size measurements, marking and genotyping procedures, and more information on population structure in space and time as well as statistical analyses are provided by Christiansen (2009), Arioli et al. (2010), Christiansen et al. (2010), and Jakob et al. (2010).

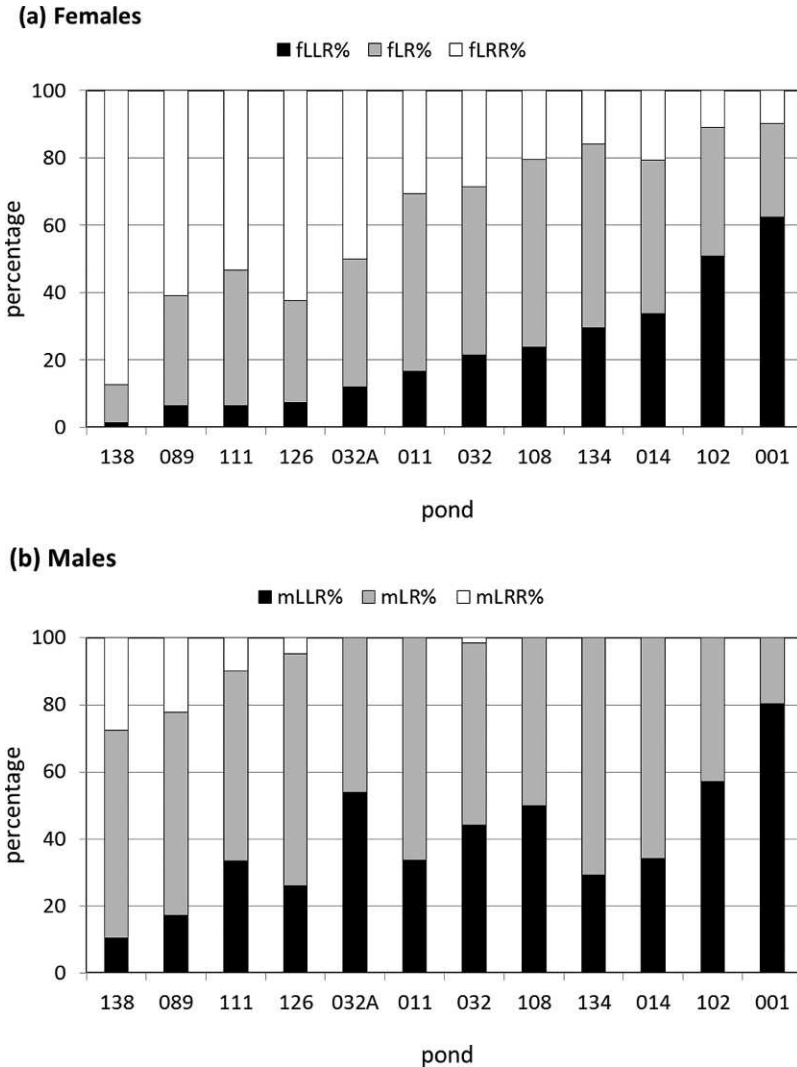


FIG. 2.—Genotype ratios in (a) female and (b) male adults of *Pelophylax esculentus* from 12 ponds in southern Sweden, collected over a period of 3 yr (2002–2004). Ponds are ordered according to increasing LLR proportions in females. Based on data by Jakob et al. (2007).

#### Age and Sex Determination

Metamorphs (i.e., individuals that have emerged from clutches laid in the year of data collection) and yearlings (i.e., individuals that have survived one winter) can easily be aged on the basis of a combination of body size and coloration, but they do not allow phenotypic sex determination. Conversely, adult individuals ( $\geq 2$  yr) can easily be sexed on the basis of the presence (males) or absence (females) of vocal sacs

and thumb pads, but determining their age requires skeletochronological analyses of toe clips. The method is based on the premise that the bone grows by an appositional process during which new material is externally laid upon the older tissue. When a transverse section through the bone is examined, a series of hematoxylinophilic, narrow rings, running parallel to the outer surface of the bone and separated from each other by broad bands, become apparent.

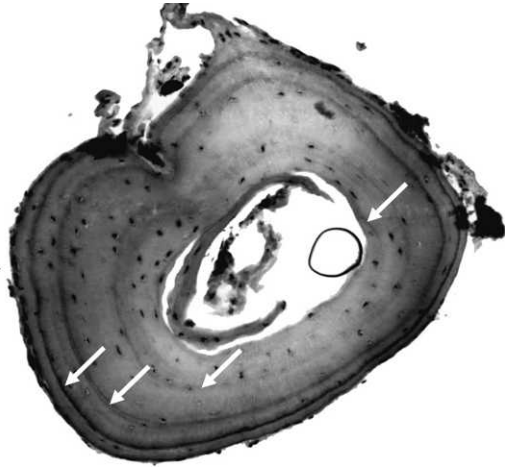


FIG. 3.—Transverse section (14- $\mu$ m thick) at the diaphysal level of a phalanx from a 4-yr-old *Pelophylax esculentus*. Ehrlich hematoxylin staining. White arrows indicate lines of arrested growth (LAG).

These narrow rings mark periods when no growth occurs, whereas the broad bands represent periods of growth. Hence, one annual layer consists of one growth zone plus its peripheral ring, which is referred to as “adhesion line” (Klevezal and Kleinberg, 1969) or “line of arrested growth” (LAG, Gibbons and McCarthy, 1983; Fig. 3).

We analyzed 576 toe clips collected by M. Arioli and C. Jakob in 2002 and 2003 and stored at the University of Zurich at  $-80^{\circ}\text{C}$  until analysis in 2004.

Following the methods of Castanet and Smirina (1990) and Miaud et al. (1993), skin and muscles were removed from the phalanges, which then were decalcified in 3% nitric acid for 6 h and subsequently washed in tap water for 12–24 h. After embedding with Tissue-TEK<sup>®</sup> O.C.T.<sup>™</sup> compound (Sakura) in disposable, peel-away, truncated embedding molds with a 12  $\times$  12-mm base (polysciences), the phalanges were sectioned transversally in the mid-diaphysal region at a thickness of 14  $\mu$ m with a freezing microtome (Microm HM 550, Walldorf, Germany). Thereafter, they were stained with Ehrlich’s eosin–hematoxylin (Fluka) solution for 20 min. After watering for 2 h, the sections were put on a microscopic slide and Aquatex<sup>®</sup> (VWR), an aqueous mounting agent, was added. We used specially coated

slides that are charged electrostatically (SuperFrost<sup>®</sup> Ultra Plus slides, Menzel-Gläser GmbH and Co KG, Germany) and therefore adhere frozen tissue sections. LAGs were counted under a light microscope (Zeiss) using  $\times 100$  and  $\times 400$  magnification.

#### Sample Sizes and Data Analysis

We analyzed age, body size, and genotype for 899 individuals: 191 metamorphs, 132 yearlings, and 576 adult frogs for which we also determined the sex. For the adults, we originally had aimed for  $\geq 10$  individuals/pond for each genotype–sex combination. However, in some ponds one genotype or the other was rare and sex ratio among LRR frogs was strongly skewed toward females. Therefore, the target sample size was achieved in only 44 of the 72 combinations (3 genotypes  $\times$  2 sexes  $\times$  12 ponds). One individual that was 9 yr old was excluded from the analysis because of its outlier character. For investigating the effects of age and genotype on body size (SVL) we performed two analyses of variance (ANOVAs). In the first, we included all seven age classes (from metamorphs to 6-yr-old individuals); for the second analysis we considered only those individuals for which the sex was known (Age Classes 2–6) and included sex as an additional factor.

Potential effects of environmental factors on age and SVL (Prediction 1) were tested with a multivariate ANOVA that related pond-specific average age and SVL (dependent variables) to the following eight ecological variables: temperature, dissolved oxygen, pH, pond size, amounts of submerged and floating pond vegetation, and proportions of forest and wetland area within 20 m of the pond. Selection of these variables from a larger set of measured factors was based on their effects found in other studies (cf. Table 2). For details of the measurement see Jakob et al. (2010). To control for the effects of genotype and sex, these two variables and their interaction were also included in the analysis.

To investigate whether pond-specific genotype ratios are related to performance and survival (Prediction 2), we correlated geno-

type ratios in the 12 ponds with average age, respectively SVL, in the same ponds. This was done separately for all genotype  $\times$  sex combinations, except for LRR males that were excluded from the analysis for two reasons. First, in each pond one genotype  $\times$  sex combination is not independent because it follows directly from the sum of the others. Second, LRR males were very rare; in some ponds they did not occur at all, in others the caught numbers were too small for calculating reliable age and SVL averages. Data analysis was done using SYSTAT 11.0 for Windows (SPSS Inc., 2002).

## RESULTS

### *Reliability of Skeletochronology for Estimating Age*

We tested the reliability of aging through skeletochronology by comparing the number of LAGs in 29 individually marked frogs that were caught in more than 1 yr. We expected an increase by one LAG in frogs that were caught in consecutive years and an increase by two LAGs in individuals with a time span of 2 yr between the first and the second catch. Twenty-three of the 29 recaptured individuals met these expectations (= 79%). The increase by two LAGs was found in all five frogs caught in 2002 and again in 2004. The increase by one LAG was detected in 18 of the 24 frogs caught in 2003 and 2004. In the six toe-clip sections that did not meet the expectation an additional ring was missing. Recatching of these six frogs tended to be earlier than that of frogs with the expected one or two additional bands (ANOVA:  $F_{2,26} = 2.72$ ,  $P = 0.084$ ): 0 LAG =  $16.50 \pm 0.837$ , 1 LAG =  $22.94 \pm 6.99$ , and 2 LAGs =  $24.40 \pm 7.79$  (mean recatching days counted from May 1,  $\pm 1$  SD). However, this tendency to underestimate age very early in the season did not affect our overall results. An ANOVA relating age to catching period (one or two), sex (male, female), and genotype (LR, LLR, LRR) explained only 1.6% of the variance, and none of the three independent variables and their two- and three-way interactions was significant (all  $P \geq 0.138$ ). Hence, there was

no catching period-related bias in age estimation with respect to genotype and sex.

### *Body Size and Age in Relation to Genotype and Sex*

*Body size.*—Overall, the three genotypes show similar growth curves (Fig. 4), with fast growth until Age Class 2 and decreasing growth rates in subsequent years. Yet, the precise pattern differs, as indicated by a significant age  $\times$  genotype interaction (Table 3a). Among metamorphs and 1-yr-old frogs, diploids (LR) are significantly smaller than triploids (LLR, LRR; metamorphs:  $F_{1,189} = 6.83$ ,  $P = 0.001$ ; Age Class 1:  $F_{1,130} = 4.26$ ,  $P = 0.041$ ). In Age Class 2 the difference disappears ( $F_{1,283} = 0.01$ ,  $P = 0.907$ ) and there is a switch leading to the size differences  $LLR < LR < LRR$  that persist from Age Class 3 through all higher classes. Although SVL correlates significantly with age in all three genotypes ( $r_{LLR} = 0.90$ ;  $P_{LLR} < 0.001$ ;  $r_{LR} = 0.93$ ;  $P_{LR} < 0.001$ ;  $r_{LRR} = 0.95$ ;  $P_{LRR} < 0.001$ ), body size variation at each age is too big for unambiguous aging (cf. SDs in Fig. 4). From Age Class 2 on, males and females can be distinguished and, hence, sex could be included in the analysis of body size. The result of the respective ANOVA (Table 3b) confirms the significant effects of age and genotype on SVL (cf. Table 2a). In addition, it reveals a significant effect of sex, with females being larger than males. Since none of the two-way interactions is significant, this sexual size dimorphism in adult frogs is independent of genotype and age.

*Age distribution.*—Since only adult frogs were sampled systematically the analysis of the age distribution was restricted to individuals from Age Classes 2–6 (Fig. 5). Within this range, the overall distribution differed neither between males and females ( $P = 0.669$ ) nor in any pair-wise comparison of the three genotypes (all  $P > 0.125$ ; Kolmogorov–Smirnov two-sample tests with two-sided probabilities).

### *Pond-Specific Age and Size in Relation to Ecological Conditions*

Only two of the eight tested ecological variables were found to be important (Table

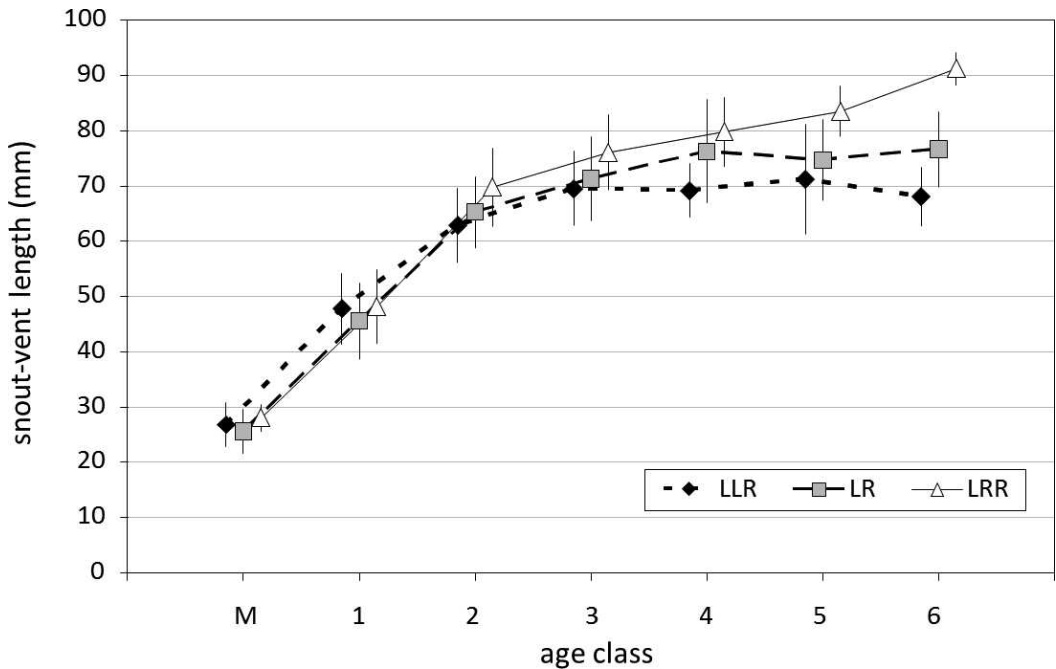


FIG. 4.—Growth curves for diploid (LR) and two types of triploid (LLR, LRR) *Pelophylax esculentus* from metamorphosis (Age Class M) to Age six (measured in years). Shown are age-specific means with standard deviations. To improve clarity, values for triploids have been slightly displaced from the real age class.

4): pond size exerted a significant positive effect on both age and SVL, whereas water temperature had a significant positive effect on SVL alone. The analysis also confirmed that genotype and sex affect body size, but not age.

*Relationship between Pond-Specific Age and Size and Genotype Ratio*

When mean age of adults ( $\geq$  Age Class 2) was related to pond-specific genotype proportions, no correlation was found for any of the five tested genotype  $\times$  sex combinations

TABLE 3.—Results from two analyses of variance relating snout-vent length (SVL) to various factors and their two-way interactions for *Pelophylax esculentus*. Analysis (a) considers all age classes but ignores sex, which was unknown for metamorphs and 1-yr-old frogs. Analysis (b) is restricted to Age Classes 2–6 for which sex is known. Significant results are shown in bold.

Source	Sum of squares	df	F ratio	P
(a)				
<b>Genotype</b>	<b>2575.1</b>	<b>2</b>	<b>32.87</b>	<b>&lt; 0.001</b>
<b>Age</b>	<b>246,382.8</b>	<b>6</b>	<b>1048.40</b>	<b>&lt; 0.001</b>
<b>Genotype <math>\times</math> age</b>	<b>1842.1</b>	<b>12</b>	<b>3.92</b>	<b>&lt; 0.001</b>
Error	34,389.5	878		
(b)				
<b>Genotype</b>	<b>451.8</b>	<b>2</b>	<b>6.93</b>	<b>0.001</b>
<b>Sex</b>	<b>780.6</b>	<b>1</b>	<b>23.96</b>	<b>&lt; 0.001</b>
<b>Age</b>	<b>1139.0</b>	<b>4</b>	<b>8.74</b>	<b>&lt; 0.001</b>
Genotype $\times$ age	178.2	8	0.68	0.706
Sex $\times$ age	61.0	4	0.47	0.759
Genotype $\times$ sex	44.0	2	0.68	0.510
Error	7069.2	217		



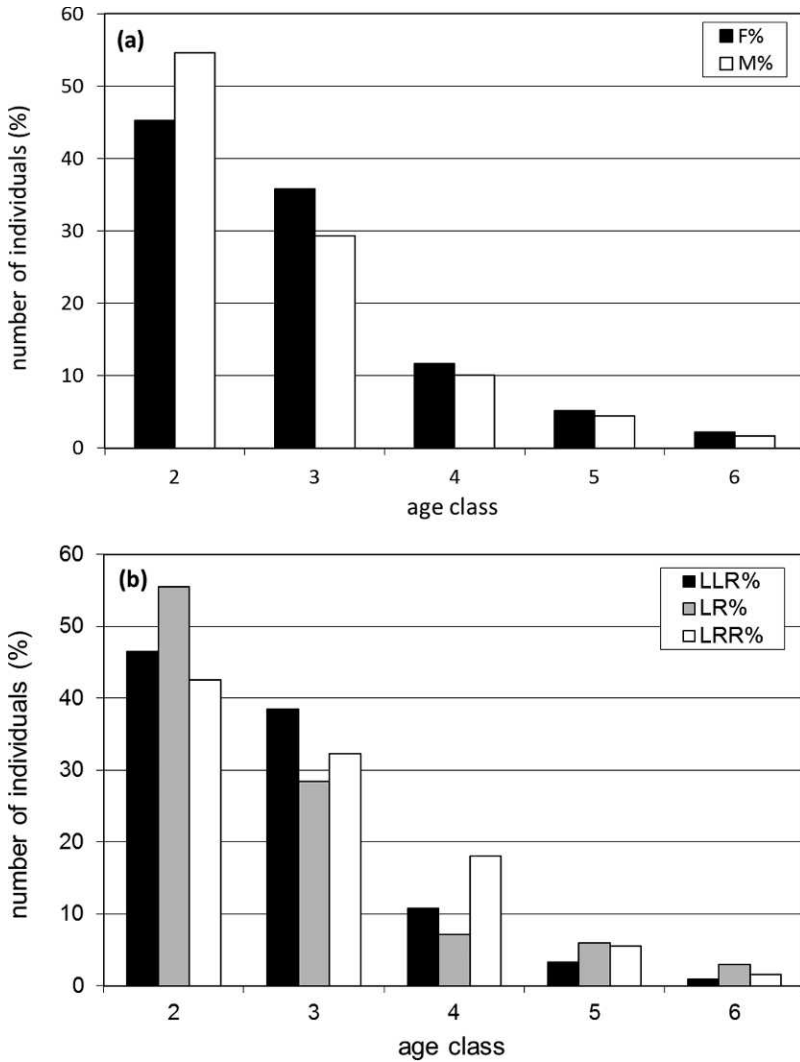


FIG. 5.—Age distribution among 576 adult *Pelophylax esculentus* in relation to sex (a) and genotype (b). For definition of age classes see Fig. 4.

( $P_{LLR,F} = 0.176$ ;  $P_{LR,F} = 0.552$ ;  $P_{LRR,F} = 0.528$ ;  $P_{LLR,M} = 0.249$ ;  $P_{LR,M} = 0.691$ ). There was also no correlation between average body size and pond-specific genotype proportions ( $P_{LLR,F} = 0.952$ ;  $P_{LR,F} = 0.210$ ;  $P_{LRR,F} = 0.218$ ;  $P_{LLR,M} = 0.165$ ;  $P_{LR,M} = 0.514$ ). Thus, there is no indication that differences in genotype composition are related to age structure and body size. Hence, different compositions do not seem to result from differences in performance and survival that arise from differences in ecological conditions.

DISCUSSION

*Reliability of Skeletochronology for Estimating Age*

When ageing the same individually marked frogs repeatedly at 1- or 2-yr intervals, the expected increase by one or two additional LAGs was detected in 23 of the 29 investigated frogs (79%). Hence, overall, skeletochronology seems to be an appropriate technique for age determination in *P. esculentus*. This is consistent with similar results from other studies on various frog species (Francillon

TABLE 4.—Results from a multivariate analysis of variance relating pond-specific average age and snout-vent length (SVL) to various genotype, sex, their interaction, and various ecological factors for *Pelophylax esculentus*. Multivariate test statistics are shown for all effects, univariate test statistics for age, and SVL only when significant (shown in bold). Ponds where age and SVL averages are based on fewer than four individuals were excluded from the analysis.

Effects	Multivariate test statistics				Age		SVL	
	Wilks' $\lambda$	<i>F</i>	df	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
<b>Genotype</b>	<b>0.742</b>	<b>3.373</b>	<b>4,84</b>	<b>0.013</b>			<b>4.052</b>	<b>0.024</b>
<b>Sex</b>	<b>0.829</b>	<b>4.336</b>	<b>2,42</b>	<b>0.019</b>			<b>8.147</b>	<b>0.007</b>
Genotype $\times$ sex	0.907	1.053	4,84	0.385				
<b>Temperature</b>	<b>0.883</b>	<b>2.779</b>	<b>2,42</b>	<b>0.074</b>			<b>5.393</b>	<b>0.025</b>
Oxygen	0.921	1.808	2,42	0.176				
pH	0.985	0.325	2,42	0.724				
<b>Pond size</b>	<b>0.720</b>	<b>8.152</b>	<b>2,42</b>	<b>0.001</b>	<b>15.241</b>	<b>&lt; 0.001</b>	<b>10.776</b>	<b>0.002</b>
Submerged vegetation	0.940	1.348	2,42	0.271				
Floating vegetation	0.954	1.022	2,42	0.369				
Forest area	0.950	1.094	2,42	0.344				
Wetland area	0.966	0.732	2,42	0.487				

and Castanet, 1985; Driscoll, 1999; Cogalniceanu and Miaud, 2003; Socha and Ogielska, 2010).

There are, however, a number of pitfalls in skeletochronology that can lead to wrong age estimation. Overestimation can result from double LAGs (i.e., two contiguous bands) that are due to nonperiodic rest lines within years. They are difficult to detect and to interpret in terms of their determinism (Castanet and Smirina, 1990). However, if present, they should be visible in the majority of individuals from a given locality. This was neither observed by us nor in another water frog study (Socha and Ogielska, 2010). Hence, the likelihood of overestimating age seemed to be fairly low. Age underestimation can result from endosteal resorption or sampling too early in the season. Endosteal resorption starts from the inner surface of the bone, widens the marrow cavity, and destroys a certain number of LAGs (Leclair, 1990; Guarino et al., 1995). However, according to Francillon and Castanet (1985), Rozenblut and Ogielska (2005), and our own results in *P. esculentus*, such resorption is an unlikely reason for age underestimation because usually only parts of LAGs get resorbed. Early sampling seems to be a more plausible explanation for not detecting additional bands in six frogs. LAGs are deposited in the outer membrane of the bone (periosteum) during hibernation, and right after hibernation, the perimeter of the bone is equal to the latest LAG (Rozenblut

and Ogielska, 2005). As a result, detecting the outermost LAG very early in the season is more difficult than later on when further bone material has been added to the periosteum. Since recatching dates tended to be earlier for the six frogs with seemingly no additional layers than for the 23 frogs where the expected bands were found, we may have overlooked the most recent LAGs in some early samples. However, this temporal effect on age determination did not affect our main results, since there was no catching period-related bias in age estimation with respect to genotype and sex.

#### *Body Size and Age in Relation to Genotype and Sex*

Overall, body size measured by SVL increased with age in a logarithmic fashion for both sexes and all three genotypes. Growth rates were high during the first 2 yr and then declined; yet, SVL continued to increase from year to year, supporting the notion that amphibians presumably have indeterminate growth (Duellmann and Trueb, 1986; Halliday and Verrell, 1988). The lower growth rate from Age Class 2 on is probably related to reproductive investment once sexual maturity has been reached. In northern populations, like ours, males start to reproduce after their second or third hibernation and females after their third hibernation (Fog et al., 1997). The growth rate decline was also reflected in the LAG pattern, with annual lines lying increas-

ingly closer after two to three hibernations. According to Kleinenberg and Smirina (1969), this happens after the individual reaches sexual maturity.

Although growth curves for the three genotypes are similar, they are not identical but gradually lead to size difference among adults. Among metamorphs and 1-yr-old frogs, the diploid LR are smaller than the triploid LLR and LRR. This difference disappears at age two where we find a switch to the size relationship that persists through all subsequent classes:  $LRR > LR > LLR$ . The size differences in the first two age classes are probably due to egg size differences, with haploid eggs (from which LR emerges) normally being smaller than diploid eggs (the origin of LLR and LRR; Uzzell et al., 1975; Berger and Roguski, 1978; Berger, 1979; Berger et al., 1986). Such egg size differences can lead to corresponding size differences in freshly hatched metamorphs (Günther, 1990), and size of metamorphosis positively affects size at later stages (Altwegg and Reyer, 2003). But with increasing age, size is more and more determined by the genotype. Given that the hybrid size is intermediate between the size of its parental species ( $LL < LR < RR$ ), it is not surprising that SVL of adult LR lies between that of LLR and LRR. The same order was found (Christiansen and Reyer, 2011) for adults in all-hybrid populations from a much larger geographical area, but the authors had no data on age-related body size.

With slightly later female than male maturity (Berger, 1970; Fog et al., 1997), one would expect the decline of growth to occur later in females than in males. However, given the lack of information on the sex of frogs from the first two age classes and the large body size variation at older ages, we were not able to test this prediction. We can only say that among adults of all three genotypes, females are significantly larger than males, a result also found by Christiansen and Reyer (2011). However, due to the large size variation in all age classes, SVL is unsuitable for sexing frogs or determining their genotype.

The sexual size dimorphism with larger female than male size that we observed in all three genotypes of the pure *P. esculentus*

populations is found in ca. 90% of anuran species (Shine, 1979) and can be pronounced in frogs (Duellmann and Trueb, 1986). A comparative analysis by Monnet and Cherry (2002) suggests that the dimorphism mainly results from differences in the age structure between the sexes in breeding populations. In species like anurans, where growth is indeterminate and female fecundity correlates with body size, selection may lead to delayed reproduction and energetic investment in females. Therefore, and because it is usually the breeding males that are particularly vulnerable to predation (e.g., Lemckert and Shine, 1993), we would expect females to live longer than the early-reproducing males (Monnet and Cherry, 2002). In our study, we could not confirm that. There was no age difference between the sexes for any of the three genotypes (Fig. 4).

#### *Ecology, Individual Performance, and Population Structure*

While statistically controlling for the above-discussed effects of genotype and sex, we also tested whether pond morphology (size, vegetation in and around the pond) and physico-chemical water conditions (temperature, oxygen, pH) influence age and body size (Table 4). Independent of genotype and sex, frogs in larger ponds were, on average, older and larger than frogs in smaller ponds, and body size also increased with water temperature. The other ecological variables exerted no significant influence on growth and survival, although previous studies had shown that they affect the composition of both mixed parental-hybrid and all-hybrid populations (see Table 2). This leads to the conclusion that ecology is unlikely to exert its influence on population structure via individual performance. The conclusion is supported by our finding that across the 12 study ponds there was no significant relationship between the proportion of a particular genotype  $\times$  sex category and the average age and size of the individuals belonging to that category. Thus, our results do neither support Prediction 1 that average age and body size should be positively related to those environmental factors that enhance the relative frequency of a particular genotype, nor do they support Prediction 2 that in

each pond individuals of the dominating genotype should, on average, be older (i.e., survive better) and bigger than individuals of the rarer genotypes. The same conclusion was reached by Christiansen et al. (2010), who correlated the mean proportion of a genotype (within sex) with survival rate (within pond) estimated by CMR of a period of 6 yr. The authors found no evidence that “differences in survival among genotypes produce the differences in adult genotype composition observed among ponds.”

How can we explain this discrepancy between expected and observed results if we assume different adaptations for the L and R genome? There are several not mutually exclusive explanations. First, given that in our pure hybrid populations all frogs carry both genomes and only differ in the L:R ratios (2:1 in LLR, 1:1 in LR, 1:2 in LRR) it is not surprising that some ecological factors affect hybrid-type ratios less than ratios of LL and RR in populations containing the parental species. This, for instance, is true for physicochemical water parameters. With respect to these factors, there is no evidence for a genome dosage effect and all three hybrid types from EE systems seem to tolerate the same wide range of conditions as LR hybrids in LE and RE systems do (Holenweg Peter et al., 2002; Jakob et al., 2010). Second, even those factors that influence LLR:LRR ratios in all-hybrid populations in the same way as LL:RR ratios in parental species populations usually explain only a small proportion of the variation in population structure. This is illustrated, for instance, by the large scatter of data points in plots of genotype proportions against pond size and surrounding vegetation, respectively, for all-hybrid populations in southern Sweden (Fig. 4 in Jakob et al., 2010). Third, depending on the geographical area, the same ecological factor can influence genotype proportions differently, which may be due to different local adaptations. An example comes from the different effects of pond-surrounding forest on genotype proportions in all-hybrid populations (cf. Table 2): Increasing forest proportions shifted the genotype ratios from LRR to LLR in German ponds (Christian-

sen and Reyer, 2011), but increased the LR ratio in Swedish ponds (Jakob et al., 2010). Fourth, fitness advantages of different hybrid types can vary with the specific set of environmental conditions (Semlitsch et al., 1997). In ponds, where these conditions change with time, recorded genotype ratios may represent a snapshot of a fluctuating situation, rather than a pond-specific pattern.

These examples strongly suggest that performance of individuals and structure of populations are not determined by a small fixed set of ecological factors. Instead, the effect of each factor depends on complicated interactions of genetic constitution and several abiotic (e.g., physicochemical) and biotic factors (e.g., vegetation, competitors, predators). Since this network varies from pond to pond and also between experimental and natural systems, the limited explanatory power of the tested ecological variables for growth, survival, and population structure may not be too surprising.

As an alternative to the ecological selection hypothesis, Christiansen et al. (2010) have recently proposed the gamete pattern hypothesis to explain the persisting differences in genotype proportions among ponds. According to this hypothesis, genotype frequencies in a pond do not change gradually with age due to selection. Instead the genotype that is dominant among adults is dominant already in the egg stage, with pond-to-pond differences arising from different gamete production patterns of LLR, LR, and LRR males and females (cf. Table 1). The authors monitored genotype proportions from eggs, through tadpoles and metamorphs to 1-yr-old frogs in three ponds that differed markedly in the proportion of LLR, LR, and LRR among adults. As in their CMR study, they found no support for the selection hypothesis (predicting a gradual change in ratios with age) and only weak support for the gamete pattern hypothesis (predicting constant genotype ratios). Thus, at present, the factors shaping genotype proportions in all-hybrid populations (and other water frog breeding systems) are not well understood and further investigations are needed.

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