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Ploidy composition in all-hybrid frog populations in relation to ecological conditions

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ABSTRACT

Question: What explains the differences in ratios of diploid (LR) and two types of triploid frogs (LLR, LRR) among all-hybrid frog populations?

Hypothesis: Ecological conditions favouring one (LL) or the other (RR) parental species also favour those triploids that carry two copies of the respective genome (dosage effect), whereas diploids dominate under intermediate conditions.

Organism: European water frog (*Pelophylax esculentus*).

Field site: Thirty-four natural ponds in the province of Skåne, southern Sweden.

Methods: We caught more than 3000 frogs, determined their genotypes with microsatellites, and related the ploidy composition to several uncorrelated ecological parameters, including pond morphology, vegetation, and physical and chemical water parameters.

Conclusions: We found a shift from predominantly LLR in small isolated ponds to more LRR in large wetland ponds. This parallels the preferences of the parental species LL and RR for small and large bodies of water, respectively. The effects that pond vegetation and physico-chemical water parameters exert on the parental species were not found in all-hybrid populations. This suggests that environmental parameters affect the genotype composition of all-hybrid populations less than populations containing the parental species. Pond-to-pond differences in LR, LLR, and LRR proportions seem to be better explained by differences in gamete production and thus inheritance patterns.

Keywords: dosage effect, gamete production, hybridogenesis, *Pelophylax esculentus*, ploidy.

INTRODUCTION

The occurrence of a species and the fitness of its members in a specific habitat typically depend on multiple abiotic and biotic factors that define the species' ecological niche. Although the distributions of different, sometimes even closely related, species can overlap, their stable co-existence in the same habitat is only possible if their realized niches differ.

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However, as a result of the overlap in species distribution, natural hybridization is quite common in both plants and animals (reviewed by Arnold, 1997; Mallet, 2005).

Despite the increasing number of studies investigating hybrid fitness, the ecological and evolutionary importance of hybrid taxa is still a contentious issue, especially among zoologists. While hybridization in plants 'has been shown to be one of the most creative forces for the evolution of earth's biodiversity' (Wissemann, 2007), hybrid taxa were long considered evolutionary dead-ends among animals because they have been shown (or assumed) to be less fit than their parental genotypes (see reviews by Arnold, 1997; Seehausen, 2004). However, in certain habitats, animal hybrids can also be fitter than their parental species [bounded superiority model (Moore, 1977; Arnold, 1997; Barton, 2001)]. One of the explanations is that the assembly of two different parental genomes allows hybrids to be successful in a wider range of ecological conditions than each of the parental species (e.g. Gao *et al.*, 2009), and/or the new gene combination may give rise to altered morphological, physiological, and behavioural traits that adapt the hybrid to novel environments (e.g. Johansen-Morris and Latta, 2008). Both broader tolerance and novel adaptations will facilitate dispersal to and settlement in new habitats. This niche shift can make the hybrid ecologically and evolutionarily very successful and even lead to speciation (Seehausen, 2004). The potential for selection on hybrids is further increased where hybrid performance differs as a result of different levels of ploidy and hence gene expression. Triploid hybrids, for instance, are often (but not always) more similar to the parental species whose genome they carry in two copies with the result that niches may differ between diploids and different types of triploids (Doeringsfeld *et al.*, 2004; Turcotte *et al.*, 2008).

Such combinations of different genomes in different numbers can increase genetic diversity even in taxa with clonal and hemiclonal reproduction, which are generally believed to be at a selective disadvantage due to reduced genetic diversity available for adaptation and the risk of accumulating deleterious mutations through Muller's ratchet (Muller, 1964). Yet, several hybrids with clonal and hemiclonal reproduction are, at least ecologically, quite successful in the sense that they occupy extensive geographical ranges and maintain large populations. Typical examples include parthenogenetic, gynogenetic, and hybridogenetic taxa among fishes, amphibians, and reptiles, which are all of hybrid origin (Dawley and Bogart, 1989). While parthenogenetic taxa can reproduce independently and thus occupy their own ecological niches, gynogenetic and hybridogenetic hybrids depend on sperm from their parental species for successful reproduction. Therefore, they must compromise between niche separation from their parental species to reduce competition and niche overlap to allow mating with the parental species.

This need for a compromise also applies to cases such as the one presented here, where maintenance of all-hybrid populations depends on matings between individuals with different ploidy levels. We investigated how the conflicting demands of inter-type mating and niche separation affect the composition of water frog populations that consist of hemiclonal diploids and two types of triploids. In particular, we tested whether ploidy ratios in all-hybrid populations can be explained through gene dosage effects of parental habitat preferences.

The European water frog system

The European edible frog, *Pelophylax esculentus* [genus *Rana* until Frost *et al.* (2006)], is an example of a highly successful hybrid taxon. Originating from repeated hybridizations between the pool frog *P. lessonae* (genotype LL) and the lake frog *P. ridibundus* (RR), *P. esculentus* (LR) is evolutionarily long-lived and geographically the most widespread water frog taxon in Europe (Plötner, 2005). Over most of its distribution range, the hybrid taxon forms stable mixed populations with one of its parental species. The reason for the co-existence lies in the hybrid's reproductive mode. *Pelophylax esculentus* reproduces hybridogenetically, also termed hemiclonally (Schultz, 1969). This mode is also known from fishes, stick insects, and salamanders (Dawley and Bogart, 1989; Mantovani and Scali, 1992). Hybridogenetic animals exclude one parental genome from the germ line prior to meiosis and transmit the other one clonally to the gametes. Hybridity is restored by mating with the parental species whose genome was excluded. While most hybridogenetic taxa are all-female and mate with males of the parental species, *P. esculentus* comes in both sexes. This opens the additional possibility of hybrid × hybrid matings, which result in offspring of a parental genotype (LL or RR, depending on which genome is transmitted); but these offspring are usually inviable, probably because of homozygosity in deleterious mutations that have accumulated on the clonally inherited genome through 'Muller's ratchet' (Muller, 1964; Vorburger, 2001).

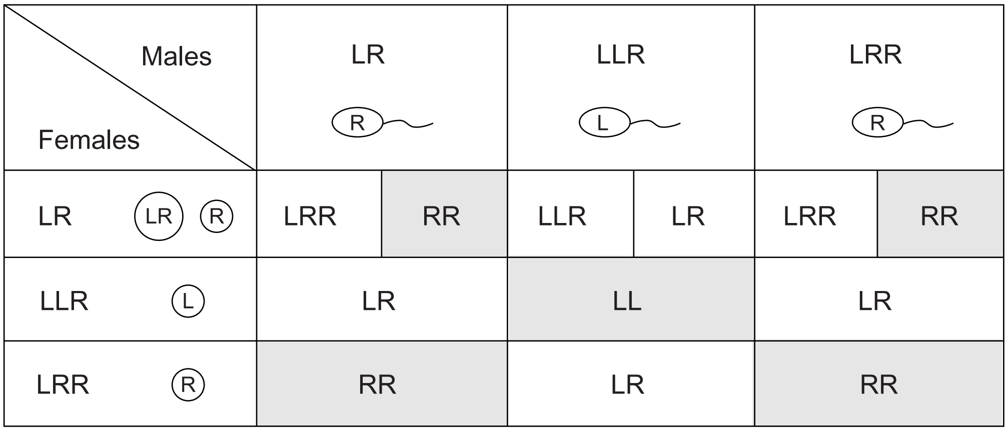
Population structure and habitat preferences in LE- and RE-systems

Due to the reproductive failure of hybrid × hybrid matings, even bisexual hybridogens like *P. esculentus* are forced into sympatry with one of their parental species. Depending on which genome is excluded (L or R), *P. esculentus* live and mate with either *P. lessonae* (LE-system) or *P. ridibundus* (RE-system). But even within each system, the composition of the mixed sexual parasite–host populations in terms of genotype proportions differs markedly. This is, in part, related to different niche preferences in both adults and larvae (summarized by Plötner, 2005).

For several ecological parameters, the hybrid's optimal niche seems to be intermediate between those of the parental species. This is indicated by both population composition in nature and experiments. Holenweg-Peter *et al.* (2002) found *P. esculentus* proportions to increase with pond size (a *ridibundus* heritage) and the percentage of vegetation under water (a *lessonae* heritage). Similarly, Pagano *et al.* (2001) describe breeding habitat partitioning of LL, LR, and RR along a gradient of river influence, with the hybrid dominating in alluvial marshes that experience a level of flooding that is intermediate between the areas occupied by *P. ridibundus* and the ones inhabited by *P. lessonae*. In experiments, tolerance of LR tadpoles to hypoxic conditions and freezing tolerance of LR adults was found to be intermediate between the tolerances of parental LL and RR individuals (Plenet *et al.*, 2000; Voituron *et al.*, 2005). Under some other conditions, hybrid larvae and/or adults proved to be superior to both parental species, suggesting the existence of heterosis effects (Hotz *et al.*, 1999; Joly *et al.*, 2008). These and other results provide plenty of evidence that L and R genomes and their combinations are adapted to different environments.

Population structure in EE-systems

An exception to the commonly occurring LE- and RE- systems is pure *P. esculentus* populations (EE-systems) in which diploid LR hybrids live and reproduce in sympatry with triploid LLR and/or LRR hybrids, rather than with the parental species (LL and/or RR). These triploids fulfil the role as sexual hosts by pre-meiotically excluding the rarer genome



Males / Females	LR Ⓡ		LLR Ⓛ		LRR Ⓡ	
LR (LR) Ⓡ	LRR	RR	LLR	LR	LRR	RR
LLR Ⓛ	LR		LL		LR	
LRR Ⓡ	RR		LR		RR	

Fig. 1. Typical gamete production in females and males for the three hybrid genotypes and offspring types arising from the nine potential mating combinations in an all-hybrid population of *P. esculentus*. Female LR regularly produce both diploid eggs (on average ~85%) and haploid eggs (15%). Triploids of both sexes mostly make haploid gametes with the genome present in two copies. There are, however, more or less pronounced deviations from these patterns (e.g. small proportions of LL eggs in LLR females, L and LR sperm in LR males), which, in part, depend on the population type (Jakob, 2007; Christiansen, 2009). Genotypes in grey boxes do not occur among the adults in the population although they are initially produced.

(R in LLR, L in LRR) and propagating the other genome after normal meiosis (Günther *et al.*, 1979; Jakob, 2007; Christiansen and Reyer, 2009) (Fig. 1). Such all-hybrid populations are mainly known from the northern distribution range of *P. esculentus* (i.e. in Northern Germany, Poland, Denmark, and Sweden) (reviewed by Günther, 1990; Plötner, 2005). Some of the earlier studies suggested that there may be two different hybrid population systems, namely LLR/LR and LRR/LR (comparable to the LE and RE systems), and that within each type proportions of diploid and triploid frogs differ among ponds. Given the fact that the parental species occupy different niches (see above), it seemed plausible to assume that – because of dosage effects – the ecological demands of the triploids also differ, with LLR being more similar to LL and LRR resembling more RR. Under this scenario, differences in composition between pure *P. esculentus* populations could result from differential fitness of the three hybrid genotypes (LLR, LR, LRR) in ponds with different ecological conditions.

In most of the early studies, however, sample sizes were small and/or genotype determination of frog types was based on (not fully reliable) morphometric rather than genetic analyses (e.g. Günther, 1975; Ebendal, 1979; Rybacki and Berger, 2001). Therefore, the real composition remained unknown. It could not even be excluded that low numbers of parental individuals were present in alleged all-hybrid populations. Moreover, proportions of the three hybrid types were not related to ecological conditions, and year-to-year stability (and thus fluctuation) was not investigated. Therefore, we (1) recorded the spatial composition and temporal stability of several all-hybrid populations in southern Sweden over a period of 3 years, and (2) related genotype composition to ecological features potentially influencing both the aquatic larval life stages of these frogs (water chemistry, physical parameters) and the adult life stages (pond morphology, land use, temperature).

MATERIALS AND METHODS

Study area

The sampling was conducted between May and August over 3 years (2002–2004) in an area located in south-eastern Skåne, southern Sweden.

Before and during the first year, we screened a total of approximately 140 ponds in the region. For the present study, we selected 34 ponds, 33 in Skåne plus one newly discovered population in Östergötland (58°06′57″N, 16°24′15″E). Pond selection was based on biological criteria such as pond size, depth, and surrounding vegetation and on practical considerations such as accessibility of the pond, number of frogs present, and feasibility of catching (e.g. related to water depth and riparian morphology). Among the 34 ponds, 12 'core ponds' were surveyed twice per season (May to July) in all 3 years. The other 22 ponds were sampled once or twice in one year only, 11 in 2002, one in 2003, and 10 in 2004. A map showing the study area and the ponds sampled, including those sampled in multiple years, can be found in Arioli *et al.* (2010).

Frog samples and genotype determination

Frogs were caught by hand during night-time with the help of a flashlight and transported to Stensoffa field station of the University of Lund. Within 24 h, the frogs were measured, weighed, and marked with a RFID PIT tag (Trovan ID101, Trovan Ltd., UK), except for animals of populations that were sampled only once. The tags were applied to allow individual recognition of frogs captured in successive years. This helped to avoid double counting (important for this study) and allowed calculation of survival rates (the subject of another study). One digit of the fourth toe was clipped for DNA analysis and stored in ethanol at −20°C until analysis. In addition, about 30–50 μl of blood was taken for flow cytometric analysis from a web vein with a heparinized capillary tube (70 μl Micro-Hematocrit Capillary Tubes, VWR International, West Chester, PA, USA) and stored in citrate buffer (D-(+)-glucose 475 mM, Sigma G8270; trisodium citrate 40 mM, Sigma-Aldrich S4641; dimethyl sulphoxide 5%, Sigma D8418; pH 7.6) at −80°C until analysis. Within 24 h, frogs were released at their capture sites. In 2002, only seemingly sexually mature frogs were collected (i.e. frogs larger than about 45 mm). In the following 2 years, also juvenile frogs entered the sample, but they were not individually marked with PIT tags. Pooled over two catches per season, mean (±S.D.) sample sizes per pond ranged from 42 ± 12 in 2002 to 98 ± 33 and 90 ± 18 in 2004 and 2003, respectively.

Genotype and ploidy determination followed the procedures described in Jakob (2007) by means of flow cytometry of nucleated red blood cells, and by gene dosage effects – that is, the relative intensity of L and R alleles amplified – in the microsatellite primers Ca1b5 (Garner *et al.*, 2000), Ca1b6, Ga1a19 (Arioli, 2007), and Res16 (Zeisset *et al.*, 2000) (see also Christiansen, 2009; Christiansen and Reyer, 2009). Samples yielding contradictory results were re-analysed and, if contradictions remained, referred to as 'mixed2n' for diploid and 'mixed3n' for triploid animals. Samples that could not be assigned to a genotype because of insufficient sample quality (tissue and/or blood) were left out of the analysis. Mosaic animals with differing DNA contents in different cells were detectable by flow cytometry only, as they showed two distinct peaks of luminescence (Jakob, 2007).

Ecological variables

Physical and chemical parameters

Dissolved oxygen (DO) was measured with an Orion DO-meter model 820 (Thermo Electron Corp., Waltham, MA, USA) in 2002 and 2003, and an HQ20 LDO sensor (Hach-Lange GmbH, Hegnau, Switzerland) in 2004. An Orion pH-meter model 230A (Thermo Electron Corp., Waltham, MA, USA) was used in 2002 and 2003 to measure pH; in 2004 we used a Hach sensIon pH electrode on an HQ 20 (Hach-Lange GmbH, Hegnau, Switzerland). Water samples (500 ml) were taken 1–4 times per season and stored at 4°C until analysis within 24 h. The following parameters were analysed: water hardness including amounts of Ca^{2+} and Mg^{2+} (°d, LCK 327), total nitrogen (TN_b, LCK 138), total carbon, total organic carbon, total inorganic carbon (TC, TOC, TIC, LCK 380 and 381), and total phosphorus (PO_4-P, LCK 349).

Temperature

Water temperature was measured in conjunction with dissolved oxygen measurements (see above) in all 3 years. In 2003 and 2004, we also installed automatic HOBO H8 temperature loggers (Onset Computer Corp., Cape Cod, MA, USA) in all core ponds, measuring temperature hourly 20 cm above and below the water line.

Pond morphology

Ponds were also characterized by 21 morphology variables, 3 of them describing size, 3 describing vegetation in and above the pond, and 15 describing land use around the pond. For size we estimated pond depths as deep (>3 m), medium (>1.5 m), or shallow (<1.5 m) and measured pond area and circumference, using GIS data and aerial photos in ArcView GIS 3.3 for Windows (© 1992–2002 ESRI Inc., Redlands, CA, USA). In terms of vegetation, we estimated the relative amount of pond covered by submerged vegetation, floating vegetation (including reed), and canopy. Land use within 5 m, 20 m, and 100 m around the ponds was estimated and assigned to five categories, labelled ‘wooded’ (including forests, trees), ‘grassland’ (meadows, pastures, lawns), ‘agriculture’ (fallows, fields), ‘wetland’ (water bodies, swamps), and ‘infrastructure’ (roads, buildings). However, because of mostly zero values in agriculture and wetland within 5 m, only land use within 20 m and 100 m was used for subsequent analyses. This reduced the pond morphology parameters to 16.

Statistical analysis

Percentage data was arcsine square-root-transformed before statistical analysis ($x' = \arcsin \sqrt{x}$) to assure a nearly-normal distribution. The relative dissolved oxygen data were an exception because values were ranging above 100% and showing a normal distribution. Non-normally distributed pond variables were log-transformed ($x' = \log(x + 1)$) to improve normality of the data set. This applied to water hardness (including amounts of Ca^{2+} and Mg^{2+}, TC, TIC, TOC, TN_b, PO_4-P) and pond area and circumference. Area and circumference were standardized (mean = 0, standard deviation = 1).

To reduce the large number of potentially correlated ecological variables to a smaller number of independent factors, each of the three sets (pond morphology, physicochemical parameters, temperature) was submitted to a separate principal components analysis (PCA, PROC FACTOR in SAS) with an orthogonal varimax rotation. Principal components (PCs) with an eigenvalue ≥ 1.00 were retained. Interpretation of principal components was based on variables with factor loadings ≥ 0.71, which, according to Comrey and Lee (1992), is considered 'excellent'.

The PCA for the 16 pond morphology variables yielded five factors accounting for 82.8% of total variation. They represent (1) forested area (which correlated positively with canopy cover and pond depth and negatively with grassland area), (2) pond size (which correlated negatively with vegetation below and above water), (3) human infrastructure, (4) agriculture, and (5) nearby wetland within 20–100 m of the pond (Table 1a). The PCA for the 12 physicochemical variables resulted in four factors, explaining 87.6% of the variation in the dataset (Table 1b). Factor (1) represents inorganic contents, factor (2) nutrients, factor (3) dissolved oxygen and pH, and factor (4) water temperature. The PCA for the eight temperature variables derived from the logged data produced two factors, accounting for 88.7% of variation in the data. These factors describe (1) absolute temperature values and (2) temperature fluctuations, respectively (results not shown). In both 2003 and 2004, the single direct temperature measurements taken during physico-chemical water sampling correlated highly with temperature measurements from the logged data (all $P < 0.05$). Therefore, the direct measurements, rather than the logged data, were used for subsequent analyses. This not only allowed us to include more than the 12 core ponds in the analysis, it also abolished the problem of lacking temperature data in two core ponds due to vandalism and logger theft.

To test for potential relationships between ecological parameters and genotype composition, we performed two stepwise linear regressions with data from 22 of the 23 ponds sampled in July 2002. In the first analysis, the dependent variable was the proportion of diploid LR in the pond. In the second analysis, the dependent variable was the proportion of LLR among the triploids. In both analyses, the independent variables were the scores from the nine principal components (five for pond morphology and four for physico-chemical variables) obtained by the above PCAs. Starting from a full model, backward elimination was performed with a significance value of <0.1 for retaining variables. Pond 032A was excluded from this analysis, because oxygen levels dropped below 5%, and neither egg clutches, tadpoles, nor newly metamorphosed frogs were encountered during our investigations.

For the core ponds, we also wished to determine whether changes in relative genotype abundance from 2002 to 2004 were related to corresponding changes in any of the physico-chemical variables and/or the initial proportions of LR and LLR among triploids in 2002. To test this, we performed linear regressions (PROC REG in SAS) on data from 11 of the 12 core ponds, again omitting pond 032A. Differences between 2002 and 2004 were standardized (mean = 0, standard deviation = 1) for all variables before analysis.

RESULTS

Overall population structure

Among the 3165 adult frogs (defined as those with a snout-to-vent length > 45 mm) that we sampled in southern Sweden, the dominating genotypes were diploid LR (41.7%), followed by triploid LLR (28.7%) and LRR (26.8%). In addition, we found some rare genotypes among adults, namely tetraploid of the LLRR type (0.3%), diploid and triploid specimens

Table 1. Results of principal component analyses with orthogonal varimax rotation of (a) 16 variables describing pond morphology and (b) 12 physico-chemical water parameters

(a) Pond morphology Variable	PC1: forest	PC2: pond size	PC3: infrastructure	PC4: agriculture	PC5: wetland
Forest (20 m)	0.938	−0.147	−0.153	−0.169	0.074
Forest (100 m)	0.933	0.064	−0.043	−0.152	0.093
Grassland (20 m)	−0.965	0.136	−0.024	0.051	−0.010
Grassland (100 m)	−0.889	−0.215	−0.122	−0.238	0.158
Canopy cover	0.657	−0.042	0.364	−0.332	0.179
Pond depth	0.614	0.350	0.099	−0.466	−0.041
Pond area	0.235	0.824	0.226	0.258	−0.066
Pond circumference	0.213	0.768	0.321	0.265	0.015
Surface vegetation	0.298	−0.696	0.382	−0.129	0.045
Submerged vegetation	0.267	−0.663	0.340	0.368	−0.085
Human infrastructure (20 m)	0.038	−0.051	0.930	−0.060	0.007
Human infrastructure (100 m)	−0.042	0.112	0.926	0.024	0.038
Agriculture (100 m)	−0.127	0.147	0.076	0.882	−0.168
Agriculture (20 m)	−0.157	0.178	−0.115	0.806	−0.018
Wetland (100 m)	−0.114	0.181	0.148	−0.017	−0.871
Wetland (20 m)	0.051	−0.205	−0.278	0.231	−0.752
Explained variance (%)	28.8	15.9	15.2	13.8	9.0

(b) Physico-chemical water parameters Variable	PC1: inorganic	PC2: nutrients	PC3: oxygen	PC4: temperature
Log water hardness (°d)	0.969	0.189	0.010	−0.079
Log total inorganic carbon ($\mathrm{mg \cdot l^{-1}}$)	0.940	0.031	−0.119	−0.183
Log Ca^{2+} ($\mathrm{mg \cdot l^{-1}}$)	0.921	0.206	0.039	−0.247
Log Mg^{2+} ($\mathrm{mg \cdot l^{-1}}$)	0.906	0.131	−0.019	0.198
Log total carbon ($\mathrm{mg \cdot l^{-1}}$)	0.717	−0.633	−0.165	0.041
Log total organic carbon ($\mathrm{mg \cdot l^{-1}}$)	0.102	−0.869	−0.124	0.168
Log total phosphorus ($\mathrm{mg \cdot l^{-1}}$)	−0.288	−0.838	0.008	0.029
Log total nitrogen ($\mathrm{mg \cdot l^{-1}}$)	−0.344	−0.776	0.126	−0.201
Dissolved oxygen ($\mathrm{mg \cdot l^{-1}}$)	−0.152	0.006	0.943	0.095
Dissolved oxygen (%)	−0.186	0.001	0.931	0.185
Log pH	0.242	0.056	0.803	−0.179
Temperature (°C)	−0.121	−0.034	0.081	0.925
Explained variance (%)	36.2	21.3	20.7	9.5

Note: Factor loadings that, according to Comrey and Lee (1992), are rated ‘good’ (>0.55), ‘very good’ (>0.63) or ‘excellent’ (>0.71) are highlighted by grey tint. Interpretation of principal components (PC) is based on variables with at least ‘very good’ loadings on the respective PC.

classified as ‘mixed’ (1.7%) because results from genotype determination methods were consistently contradictory, and ‘mosaic’ individuals (0.6%), carrying blood cells with varying genotypic content (e.g. LLR and LRR, LLL and LLR or LL and LR). With the exception of one pond where in 2004 a total of three RR females (0.1%) were found, parental genotypes (LL or RR), which can arise from five of the nine mating combinations

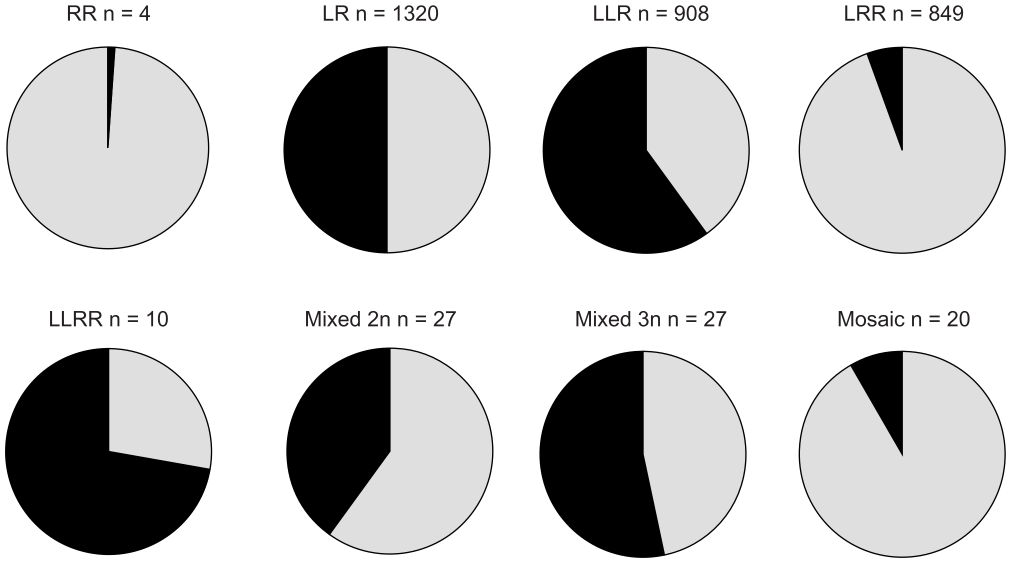
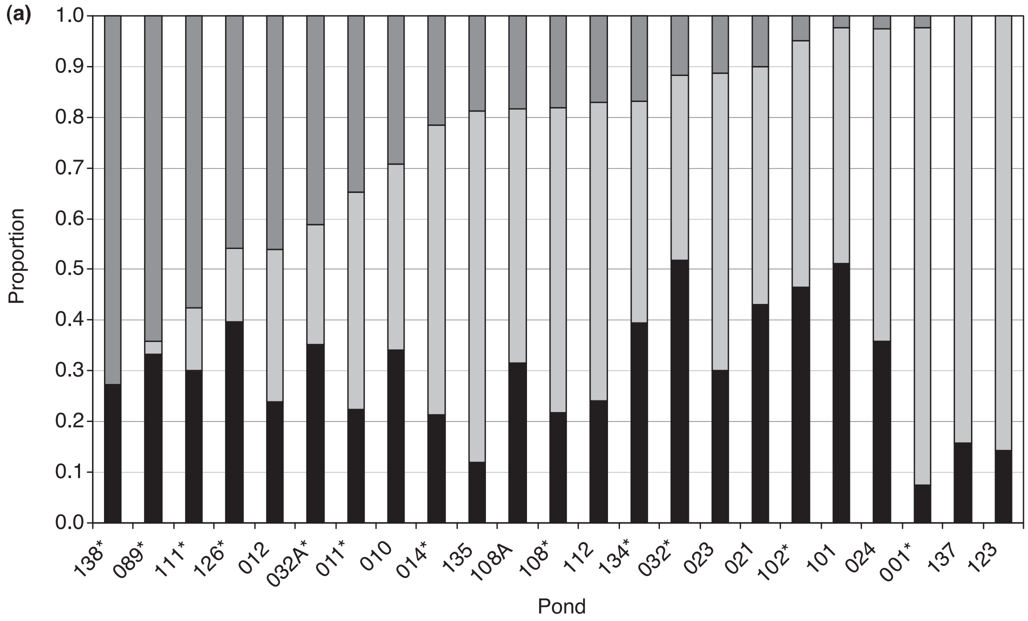


Fig. 2. Sex proportions in different genotypes (mean over all ponds, 2002–2004). While LRR, RR, and mosaic animals are almost exclusively female (grey), males (black) dominate in LLRR and, to a lesser extent, also in LLR.

shown in Fig. 1, were completely absent from adult samples. This absence arises neither from the lack of the respective mating combinations nor from a sampling bias; it is the result of a continuous decrease in parental genotypes from eggs (25% RR, 15% LL), through tadpoles (4.8% RR, 3.3% LL) to metamorphs (<1% RR, 0% LL) (Arioli, 2007). Hence, the adult water frog populations sampled in Scania are all-hybrid due to higher juvenile mortality of parental genotypes.

While adult LR and mixed 2n and 3n genotypes consisted about equally of males and females (all $P \geq 0.726$; binomial test), the sex ratios of triploids and mosaic animals were skewed (Fig. 2): LRR and mosaic frogs exhibited a strong female bias (both $P \leq 0.012$), whereas LLR animals were, to a lesser extent, biased towards males ($P < 0.001$). Sex biases may also exist in RR (only females) and LLRR (7 females, 3 males) (both $P \geq 0.136$), but sample sizes were too low to reach significance.

In 2002, the number of diploid adult LR animals in Scania varied between 7.7% and 53.1% (mean = 28.9%, S.D. = 11.6%). In only 2 of the 23 ponds sampled in that year diploid animals slightly, but not significantly, outnumbered the triploids; the other ponds were dominated by triploids (LLR and LRR genotypes combined) (Fig. 3a). With the exception of three ponds, two lacking LRR and one lacking LLR, all three genotypes occurred sympatrically in all ponds, although at varying rates. Co-existence of the three main genotypes was true also for 12 of 13 ponds sampled in 2003 and for 19 of 21 ponds sampled in 2004. In none of the three years were LR proportions significantly correlated with either LLR or LRR proportions (all $P \geq 0.123$), but in each year there was a highly significant negative correlation between LLR and LRR proportions (all $P \leq 0.002$). Hence, populations can be arranged along a triploid gradient from exclusively LLR to exclusively LRR, but the proportion of diploid LR is independent of the triploid composition.



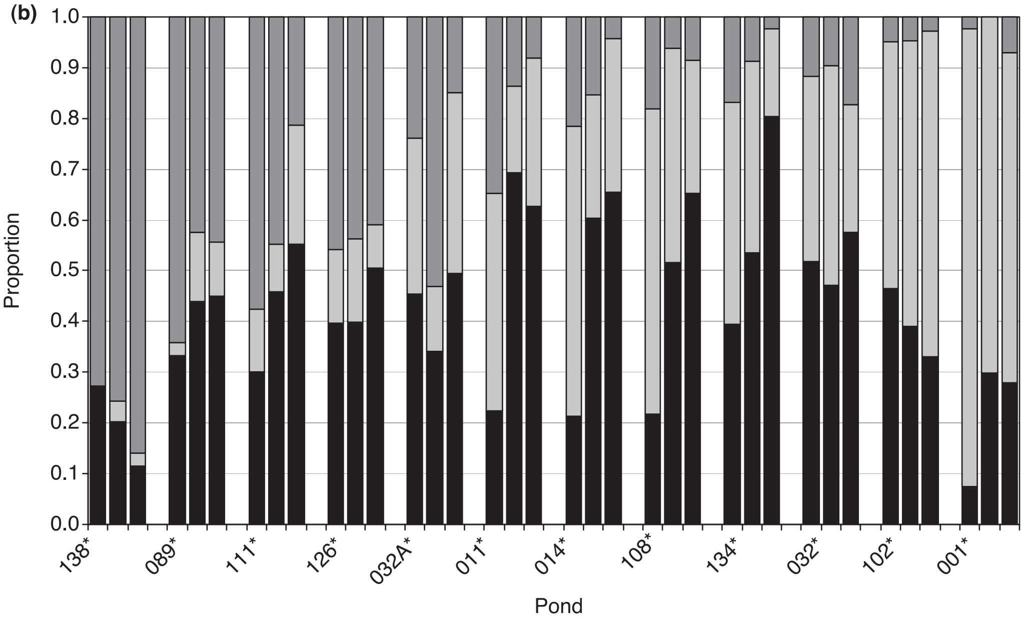


Fig. 3. Proportions of LR (black), LLR (light grey), and LRR (dark grey) in (a) 23 ponds sampled in 2002 and (b) the 12 core ponds sampled over three consecutive years (2002–2004). Data from males and females are pooled.

In contrast to these all-hybrid populations from Scania, the population in Östergötland represented a typical LE population with only diploid hybrids (LR) and *P. lessonae* (LL) individuals in both adults ($n = 17$) and juveniles ($n = 23$).

Spatial and temporal differences in genotype composition and sex ratio

Since for logistical reasons we had to sample in different years and at different times during the breeding season, the above-mentioned pond-to-pond differences in genotype composition might, in part, reflect temporal rather than spatial differences. To test for that, we subjected genotype proportions and sex ratios from the 12 core ponds to an analysis of covariance (ANCOVA) with pond and year as independent random variables and the first and second sampling during the season as repeated measures of the dependent variable. For adults, the results show significant effects of both pond ($P = 0.049$) and year ($P < 0.001$) on the proportion of diploid LR (Table 2). Overall, the LR proportion increased from 2002 through 2003 to 2004; but the change varied among ponds, as indicated by the marginally significant pond × year interaction ($P = 0.049$) (Fig. 3b). From 2002 to 2004, diploid proportions continuously decreased in some ponds (e.g. 102, 138), increased in others (e.g. 108 and 111) or showed different patterns in still others (e.g. 032 and 032A) (cf. Fig.3b). Season and its two- and three-way interactions with pond and year were not significant for either the LR proportion (all $P \leq 0.142$) or the proportion of females within the diploids (all $P \geq 0.099$). Hence, within years, the proportion of diploid LR and the sex ratio among them were not affected by sampling date.

Changes in LR proportions over the years are observed with opposite changes in the proportions of triploids, but these must not necessarily affect LLR and LRR equally. We therefore performed the same analysis for the proportion of LLR among the total number of triploids. There was no significant effect of either pond or year on LLR/LRR ratios (Table 2), indicating that changes in LR proportions affect the LLR and LRR proportions about equally, and vice versa. This further supports the above notion that the relationship between diploid and triploid proportions is similar for LLR and LRR dominated populations.

Overall, sex ratios did not vary between years for any of the three genotypes (all $P \geq 0.635$), but there were pond-specific differences between years for LR (year × pond interaction: $P = 0.025$) and between seasons for LRR (pond × season interaction: $P = 0.033$) (Table 2).

In juveniles, the proportions of diploid LR and of LLR among the triploids did not differ between either core ponds or years (all $P \geq 0.119$). However, for this age class we have data for only two years (2003, 2004), and within both years samples sizes are smaller that those for adults.

Genotype composition and changes in relation to ecological parameters

Locations of the investigated ponds ranged from open fields surrounded by wetland through agricultural and/or human settlement neighbourhoods to inside forests. This wide range of habitats (‘pond morphology’) was observed with an equally wide range of between-pond differences in other ecological conditions, including pond size (range 140–5700 m^2) and depth (shallow to deep), vegetation in and around the pond, temperature (16.6–23.6°C in the first week of June), dissolved oxygen concentration (0.4–32.4 $mg \cdot l^{-1}$),

Table 2. Results of repeated-measures ANCOVAs relating adult sex ratios in the three main genotypes (LR, LLR, LRR) and proportions of diploid LR and LLR among triploids (LLR/3n) to pond, year, and the pond × year interaction (a). Due to fewer sampling events, the pond × year interaction could not be calculated for juveniles. For adults we performed a repeated-measures analysis, since two samples per season were taken (b)

(a) Between subjects	Adult genotype proportions				Adult sex ratios						Juvenile genotype proportions			
	LR		LLR/3n		LR		LLR		LRR		LR		LLR/3n	
Effects	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Pond	2.73	**0.049**	0.90	0.570	3.32	**0.025**	1.22	0.379	3.85	**0.042**	1.29	0.349	2.99	0.119
Year	26.33	**<0.001**	3.57	0.083	0.24	0.635	0.00	0.981	0.00	0.999	0.88	0.370	0.02	0.895
Pond × year	2.73	**0.049**	0.89	0.570	3.32	0.025	1.22	0.379	3.84	**0.042**				

(b) Within subjects	Adult genotype proportions				Adult sex ratios					
	LR		LLR/3n		LR		LLR		LRR	
Effects	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Season	2.47	0.142	0.42	0.531	0.00	0.992	0.63	0.448	0.00	1.000
Season × pond	1.13	0.419	3.89	**0.014**	2.18	0.099	0.69	0.714	4.25	**0.033**
Season × year	2.47	0.142	0.42	0.531	0.00	0.992	0.63	0.447	0.00	1.000
Season × pond × year	1.12	0.420	3.89	**0.014**	2.18	0.099	0.69	0.714	4.25	**0.033**

Note: Only the 12 core ponds were included in this analysis. *P*-values <0.05 are printed in **bold**.

pH (5.35–12.20), water hardness (0.3–50.7), total organic carbon (8.8–82.4), and other physico-chemical features of the water. In studies of LE- and RE-systems, several of these factors and ranges have been shown to affect the occurrence and fitness of hybrid and parental adults and larvae differently. However, since many of these ecological features were correlated, we first reduced the original measures to fewer independent variables through PCA and then used the scores of the resulting principal components to test for the influence of ecology on genotype composition.

The stepwise linear regression of LR proportions against factor scores of the nine principal components representing inorganic contents, nutrients, dissolved oxygen and pH, water temperature, pond size, and the relative amounts of forest area, infrastructure, and agriculture within 20–100 m around the pond resulted in a significant model ($F_{1,20} = 6.882$, $P = 0.016$) with only one explanatory variable remaining: the proportion of diploids increased with the relative amount of forested area around the pond ($P = 0.016$) (Fig. 4a). The stepwise linear regression relating the proportion of LLR among the triploids against the same nine independent variables also resulted in a significant model ($F_{2,19} = 8.200$, $P = 0.003$). LLR proportions decreased with pond size ($P = 0.018$) and the proportion of wetland within 20 m ($P = 0.005$) (Figs. 4b,c).

For the 22 ponds used in the above analysis, size, vegetation, and surroundings were recorded only in 2002, since pond morphology does not change from year to year. In the 12 core ponds, however, we recorded the physico-chemical variables also in 2003 and 2004. When relating these data to pond, year. and early and late measurements within a year by means of a repeated-measures ANCOVA, we found significant differences in water hardness, total organic carbon, and oxygen between ponds (all $F_{11} \geq 2.371$, $P \leq 0.048$), but not between years and early and late measurements (Table 3). Temperature differed not only between ponds ($F_{11} = 12.087$, $P = 0.001$), but also between years ($F_1 = 74.608$, $P < 0.001$) and between early (cooler) and late (warmer) measurements, with differences from year to year (season × year interaction: $F_1 = 7.372$, $P = 0.026$). These annual temperature changes,

Table 3. Results for a repeated-measures ANCOVA testing for (a) the effects of pond and year as well as (b) season within year (2 repeats) on four physico-chemical water parameters: hardness, total organic carbon, oxygen, and temperature

(a) Between subjects	Hardness		Organic carbon		Oxygen		Temperature	
Effects	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Pond	0.09	0.765	0.24	0.636	0.29	0.599	74.61	**<0.001**
Year	96.14	**<0.001**	2.87	**0.042**	2.37	**0.048**	12.09	**0.001**
(b) Within subjects	Hardness		Organic carbon		Oxygen		Temperature	
Effects	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Season	0.08	0.776	0.51	0.490	1.17	0.294	7.37	**0.026**
Season × pond	0.083	0.777	0.51	0.490	1.17	0.294	7.37	**0.026**
Season × year	2.082	0.074	1.57	0.226	2.15	0.069	0.56	0.817

Note: Only core ponds were included in this analysis. *P*-values <0.05 are printed in **bold**.

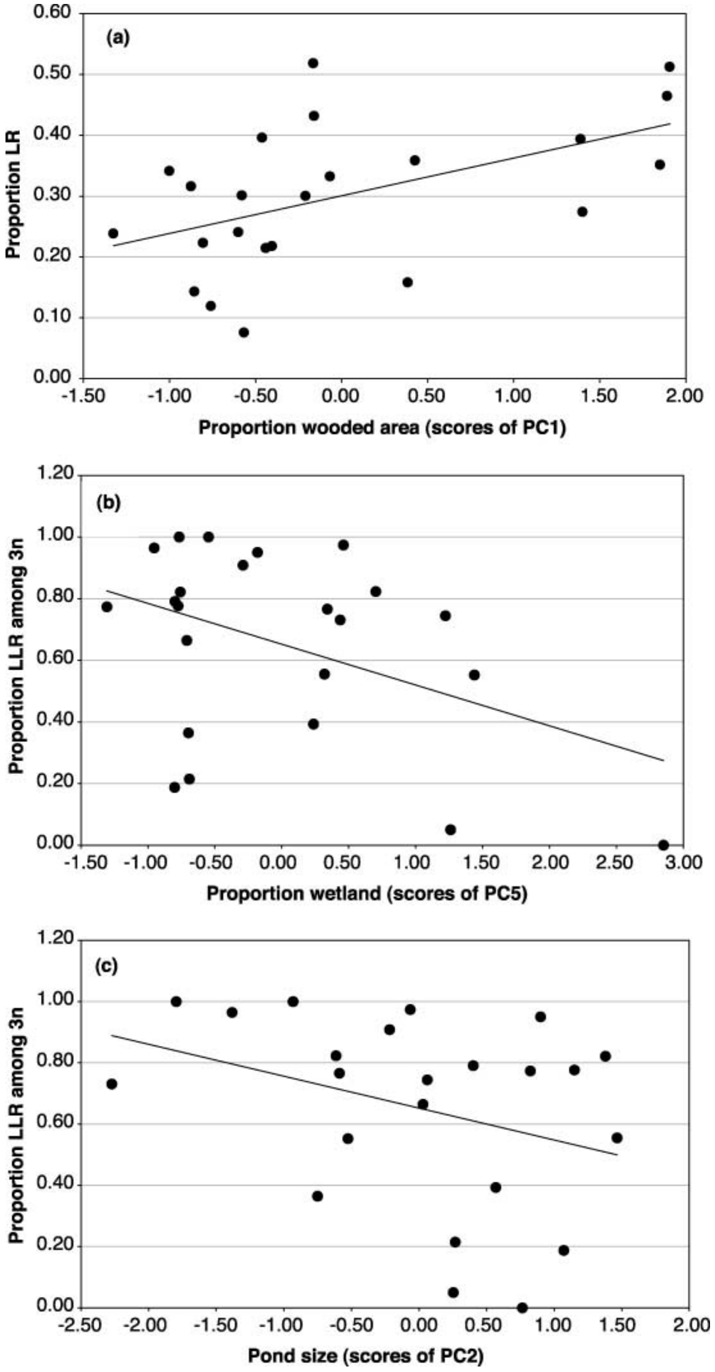


Fig. 4. Relationships between ecological conditions and proportions of LR (a) and LLR among triploids (b, c), respectively. Shown are only those relationships that remained in the final models of stepwise regression analyses (see text). Since loadings of wetland proportion on PC5 are negative (Table 1a) and hence low scores mean high wetland proportions, scores in (b) were multiplied by –1 to reverse the X-axis and let values increase from left to right as in (a) and (c).

however, did not affect the genotype proportions. When pond-specific changes in diploid proportions and LLR proportions within triploids from 2002 to 2004 were related to temperature changes in the same ponds during the same period by means of linear regression, no significant relationship emerged (LR: $t = -1.703$, $P = 0.123$; LLR/n3: $t = -0.717$, $P = 0.492$).

DISCUSSION

Composition of all-hybrid populations in space and time

Our genetic analysis of more than 3000 individual specimens shows that *P. esculentus* forms all-hybrid populations in Skåne. This had already been suggested by Ebendal (1979) but, due to low sample sizes and no genetic analyses, the author could not exclude the possibility that parental genotypes were present in low numbers. Also, he was unable to reliably distinguish between different hybrid types and described the Skåne populations as an LR/LLR system. In contrast, our study in the same area, as well as that by Christiansen *et al.* (2005) in three all-hybrid populations in Denmark, showed that LRR individuals are also quite common and that the three main hybrid genotypes (LR, LLR, and LRR) occur in most of the ponds simultaneously, although in varying proportions.

In our 12 core ponds, these proportions changed over the 3 years of our study. Overall, there was an increase in LR at the expense of triploid animals (Table 2a), but changes differed between ponds, years, and seasons (see interaction in Table 2b). These fluctuations can be due to both methodological and biological reasons. With three genotypes, two sexes, and two catching rounds per season, even a sample of 90 frogs per pond will result, on average, in only 7–8 individuals per category and sampling event; this makes calculated proportions sensitive to stochasticity. Moreover, during a season water frogs can temporarily leave the pond and return later, and genotypes may arrive unsynchronized (unpublished data), which illustrates the importance of sampling populations more than once during the season. In terms of biology, year-to-year fluctuations in genotype proportions are to be expected even when, in the long run, all-hybrid populations should be stable (Som and Reyer, 2006). This is because a high percentage of triploid parents (which produce haploid gametes) will lead to a high percentage of diploid offspring, whereas a high proportion of diploid females (mostly producing diploid gametes) will enhance the frequency of triploid offspring. These short-term fluctuations are not necessarily synchronized between ponds, as indicated by the pond × year interaction effect on LR proportions (Fig. 3b). The reasons for this asynchrony may include different starting conditions, mating stochasticity, and pond-specific generation lengths (i.e. the time from eggs to sexual maturity) as well as other factors.

In addition to the three typical genotypes we detected some exceptional ones, namely 10 symmetric tetraploids (LLRR), 54 'mixed' animals with ambiguous genotype determination, and 20 'mosaic' individuals carrying red blood cells with varying genotypic content (e.g. LLR and LLR, LLL and LRR or LL and LR). Despite their rareness (2.6% in total), they deserve special attention because they could affect the structure and dynamics of all-hybrid populations in various ways. 'Mixed' animals, for instance, may indicate introgression of L genes or genome parts into the R genome and vice versa. Symmetric tetraploids could introduce normal meiosis into the hybridogenetic system and provide a step towards the formation of a new, independent species with sexual reproduction (Vrijenhoek, 2006). Such a possibility has recently been postulated for the diploid/polyploid fish hybrid *Squalius alburnoides* system (Alves *et al.*, 1999; Pala and Coelho, 2004). Since the proportions of mixed and tetraploid animals are 6–8 times higher during early stages (egg, larvae, metamorphs) than among adults (Arioli, 2007), survival of these unusual genotypes must have been low in our ponds. They may, however, do better under exceptional conditions and then further increase the complexity of this hybridogenetic system.

Population composition in relation to ecological conditions

The above-mentioned stochastic and deterministic factors influencing genotype proportions might have impaired our success in identifying ecological causes for differences in population composition among ponds and years. Most of the measured environmental variables did not explain these differences. The only exception is pond morphology. Despite large variation, relative numbers of LR genotypes increased (and hence the triploid proportion decreased) significantly with the proportion of wooded area around the pond (scores of PC1). Among the triploids, the proportion of LLR decreased with increasing pond size (scores of PC2) and increasing wetland area around the pond (scores of PC5). This shift from predominantly LLR in small isolated ponds to more LRR in large wetland ponds parallels the preferences of the parental species LL and RR for small and large bodies of water, respectively (Fig. 2a in Holenweg-Peter *et al.*, 2002). With a slope of −0.177, the effect of pond size on the proportion of LLR animals among triploids in EE populations (Fig. 4c) is intermediate between pond size effects on LR (+0.281) and LL (−0.309) measured by Holenweg-Peter *et al.* (2002) in LE populations. This is in agreement with the expected dosage effect. For all other environmental factors there is no indication that dosage effects make niches of LRR and LLR hybrids more similar to those of the respective parental species. Rather, all hybrid types seem to possess the same broad tolerance for a wide range of ecological conditions as previously found for LR hybrids in LE- and RE-systems.

There are several, not mutually exclusive, explanations for not finding clear ecological segregation between genotypes. First, the span of ecological conditions in and around the investigated ponds was too narrow to exclude certain specialized genotypes. For the parameters that we monitored, this explanation is unlikely. The range of habitats and ecological conditions in and around the ponds was at least as wide as those in studies of LE- and RE-systems where clear differences in occurrence and fitness of LR, LL, and RR larvae and adults were found (see Introduction). There are, however, several unrecorded variables that may have affected the relative success of the different genotypes, including parasites and predators that are known to mediate competition and co-existence in other systems (Hudson and Greenman, 1998; Spaak and Boersma, 2006).

Second, in polyploids one and one does not always equal two (Otto, 2003) – that is, two copies of one genome do not necessarily shift the ecological needs of the hybrid in the direction of the respective parental species. In both plants and animals, it has been shown that polyploidy does not always lead to enhanced transcription of duplicated genes; rather, gene copy silencing may reduce transcript levels to the diploid state (Adams *et al.*, 2003; Pala *et al.*, 2008). The allelic expression pattern may even differ between genes of the same individual (Pala *et al.*, 2008). As a result, polyploid hybrids can show dosage effects for some traits, but not for others (Kearney and Shine, 2004; Turcotte *et al.*, 2008); and hybrids in general can be intermediate between parental species for some traits, parental-like for others, and transgressive (i.e. more extreme than in both parental species) for still others (Schwarzbach *et al.*, 2001; Gao *et al.*, 2009).

Third, selection regimes for mating behaviour differ between diploid/triploid all-hybrid populations (EE-system) and those where diploid hybrids live and mate with one of the parental species (LE- and RE-systems). In the latter, the hybrid depends on parental individuals for successful reproduction, but the parentals can exist without hybrids. In all-hybrid populations, however, members are mutually dependent on each other, because production of viable offspring requires that mating occurs between males and females of different ploidies (Som and Reyer, 2006). Hence, in all-hybrid populations LLR and LRR animals are forced into co-existence and mating with diploid LR, whereas in LE- and RE-systems the parental LL and RR are not, and they even should avoid mating with hybrids. This is also reflected in the mating behaviour: both LL and LR females from the LE-system prefer LL over LR males (Roesli and Reyer, 2000; Engeler and Reyer, 2001), but LLR and LR females from EE-systems exhibit no choice (Günther and Plötner, 1990; Rondinelli, 2006). Thus, niche separation from LR is likely to be enhanced by sexual selection in LL and RR, but to be reduced in LLR and LRR.

CONCLUSIONS

Given that strong ecological niche differentiation between LLR, LR, and LRR hybrids is unlikely to exist for the above-mentioned reasons and did not find clear support in this study on Swedish populations or a study that included Danish populations (Christiansen and Reyer, 2010), the question arises how the differences in all-hybrid population composition can be explained. Christiansen (2009) has recently proposed the gamete pattern hypothesis, which originated from results of her own and previous crossing experiments (Jakob, 2007) with frogs from LLR-rich, LR-rich, and LRR-rich ponds. Both studies found differences in gamete production between these ponds, especially in the proportions of L, R, and LR sperm produced by LR males (cf. legend to Fig. 1). Based on the observed gamete proportions, Christiansen (2009) calculated the equilibrium genotype proportions for the three different population types and found excellent agreement between predicted and observed LLR, LR, and LRR proportions for both male and female adults. This suggested that genetically determined gametogenesis patterns offer a better explanation for differences in genotype composition than selection through ecological conditions. However, when both explanations were tested simultaneously in juvenile stages (eggs, tadpoles, metamorphs, and one-year-old froglets), results yielded only slightly better support for the gamete than for the selection hypothesis (Christiansen *et al.*, 2010). Thus, more studies are needed before the different genotype proportions in all-hybrid *P. esculentus* populations can be satisfactorily explained.

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REFERENCES

Adams, K.L., Cronn, R., Percifield, R. and Wendel, J.F. 2003. Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-specific reciprocal silencing. *Proc. Natl. Acad. Sci. USA*, **100**: 4649–4654.

Alves, M.J., Coelho, M.M., Prospero, M.I. and Collares-Pereira, M.J. 1999. Production of fertile unreduced sperm by hybrid males of the *Rutilus alburnoides* complex (Teleostei, Cyprinidae): an alternative route to genome tetraploidization in unisexuals. *Genetics*, **151**: 277–283.

Arioli, M. 2007. Reproductive patterns and population genetics in pure hybridogenetic water frog populations of *Rana esculenta*. PhD thesis, University of Zurich.

Arioli, M., Jakob, C. and Reyer, H.-U. 2010. Genetic diversity in water frog hybrids (*Pelophylax esculentus*) varies with population structure and geographic location. *Mol. Ecol.*, **19**: 1814–1828.

Arnold, M.L. 1997. *Natural Hybridization and Evolution*. Oxford: Oxford University Press.

Barton, N.H. 2001. The role of hybridization in evolution. *Mol. Ecol.*, **10**: 551–568.

Christiansen, D.G. 2009. Gamete types, sex determination and stable equilibira of all-hybrid populations of diploid and triploid edible frogs (*Pelophylax esculentus*). *BMC Evol. Biol.*, **9**: 135.

Christiansen, D.G. and Reyer, H.-U. 2009. From clonal to sexual hybrids: genetic recombination via triploids in all-hybrid populations of water frogs. *Evolution*, **63**: 1754–1768.

Christiansen, D.G. and Reyer, H.-U. 2010. Effects of geographic distance, sea barriers and habitat on the genetic structure and diversity of all-hybrid water frog populations. *Heredity* (DOI: 10.1038/hdy.2010.37).

Christiansen, D.G., Fog, K., Pedersen, B.V. and Boomsma, J.J. 2005. Reproduction and hybrid load in all-hybrid populations of *Rana esculenta* water frogs in Denmark. *Evolution*, **59**: 1348–1361.

Christiansen, D.G., Jakob, C., Arioli, M., Roethlisberger, S. and Reyer, H.-U. 2010. Coexistence of diploid and triploid hybrid water frogs: population differences persist in the apparent absence of differential survival. *BMC Ecol.*, **10**: 14.

Comrey, A.L. and Lee, H.B. 1992. *A First Course in Factor Analysis*, 2nd edn.. Hillsdale, NJ: Lawrence Erlbaum Associates.

Dawley, R.M. and Bogart, R.P. 1989. *Evolution and Ecology of Unisexual Vertebrates*. Albany, NY: New York State Museum.

Doeringsfeld, M.R., Schlosser, I.J., Elder, J.F. and Evenson, D.P. 2004. Phenotypic consequences of genetic variation in a gynogenetic complex of *Phoxinus eos-neogaeus* clonal fish (pisces: Cyprinidae) inhabiting a heterogeneous environment. *Evolution*, **58**: 1261–1273.

Ebendal, T. 1979. Distribution, morphology and taxonomy of the Swedish green frogs (*Rana esculenta* complex). *Mitt. Mus. Natkd. Berlin*, **55**: 143–152.

Engeler, B. and Reyer, H.-U. 2001. Choosy females and indiscriminate males: mate choice in mixed populations of sexual and hybridogenetic water frogs (*Rana lessonae*, *Rana esculenta*). *Behav. Ecol.*, **12**: 600–606.

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B. et al. 2006. The amphibian tree of life. *Bull. Am. Mus. Nat. Hist.*, **297**: 8–270.

Gao, D.H., Gao, Q., Xu, H.Y., Ma, F., Zhao, C.M. and Liu, J.Q. 2009. Physiological responses to gradual drought stress in the diploid hybrid *Pinus densata* and its two parental species. *Trees Struct. Funct.*, **23**: 717–728.

Garner, T.W., Gautschi, B., Röthlisberger, S. and Reyer, H.-U. 2000. A set of CA repeat microsatellite markers derived from the pool frog, *Rana lessonae. Mol. Ecol.*, **9**: 2173–2175.

Günther, R. 1975. Zum natürlichen Vorkommen und zur Morphologie triploider Teichfrösche, *'Rana esculenta'*, L., in der DDR (Anura, Ranidae). *Mitt. Zool. Mus. Berlin*, **51**: 145–158.

Günther, R. 1990. *Die Wasserfrösche Europas*. Wittenberg: A. Ziemsen Verlag.

Günther, R. and Plötner, J. 1990. Mating pattern in pure hybrid populations of water frogs *Rana esculenta* (Anura Ranidae). *Alytes*, **8**: 90–98.

Günther, R., Uzzell, T. and Berger, L. 1979. Inheritance patterns in triploid *Rana 'esculenta'* (Amphibia, Salientia). *Mitt. Mus. Natkd. Berlin*, **55**: 35–57.

Holenweg-Peter, A.-K, Reyer, H.-U. and Abt Tietje, G. 2002. Species and sex ratio differences in mixed populations of hybridogenetic water frogs: The influence of pond features. *Ecoscience*, **9**: 1–11.

Hotz, H., Semlitsch, R.D., Gutmann, E., Guex, G.-D. and Beerli, P. 1999. Spontaneous heterosis in larval life-history traits of hemiclonal frog hybrids. *Proc. Natl. Acad. Sci. USA*, **96**: 2171–2176.

Hudson, P. and Greenman, J. 1998. Competition mediated by parasites: biological and theoretical progress. *Trends Ecol. Evol.*, **13**: 387–390.

Jakob, C. 2007. Structure and dynamics of pure hybridogenetic water frog populations. PhD thesis, University of Zurich.

Johansen-Morris, A.D. and Latta, R.G. 2008. Genotype by environment interactions for fitness in hybrid genotypes of *Avena barbata*. *Evolution*, **62**: 573–585.

Joly, P., Guesdon, V., Fromont, E., Plenet, S., Grolet, O., Guegan, J.F. et al. 2008. Heterozygosity and parasite intensity: ling parasites in the water frog hybridization complex. *Parasitology*, **135**: 95–104

Kearney, M. and Shine, R. 2004. Morphological and physiological correlates of hybrid parthenogenesis. *Am. Nat.*, **164**: 803–813.

Mallet, J. 2005. Hybridization as an invasion of the genome. *Trends Ecol. Evol.*, **20**: 229–237.

Mantovani, B. and Scali, V. 1992. Hybridogenesis and androgenesis in the stick-insect *Bacillus rossius-grandii benazzii* (Insecta Phasmatodea). *Evolution*, **46**: 783–796.

Moore, W.S. 1977. An evaluation of narrow hybrid zones in vertebrates. *Q. Rev. Biol.*, **52**: 263–277.

Muller, H.J. 1964. The relation of recombination to mutational advance. *Mutat. Res.*, **1**: 2–9.

Otto, S.P. 2003. In polyploids, one plus one does not equal two. *Trends Ecol. Evol.*, **18**: 431–433.

Pagano, A., Joly, P., Plenet, S., Lehman, A. and Grolet, O. 2001. Breeding habitat partitioning in the *Rana esculenta* complex: the intermediate niche hypothesis supported. *Ecoscience*, **8**: 294–300.

Pala, I. and Coelho, M.M. 2004. Contrasting views over a hybrid complex: between speciation and evolutionary 'dead-end'. *Gene*, **347**: 283–294.

Pala, I., Coelho, M.M. and Schartl, M. 2008. Dosage compensation by gene-copy silencing in a triploid hybrid fish. *Curr. Biol.*, **18**: 1344–1348.

Plenet, S., Hervant, F. and Joly, P. 2000. Ecology of the hybridogenetic *Rana esculenta* complex: differential oxygen requirements of tadpoles. *Evol. Ecol.*, **14**: 13–23.

Plötner, J. 2005. *Die westpaläarktischen Wasserfrösche*. Bielefeld: Laurenti-Verlag.

Roesli, M. and Reyer, H.-U. 2000. Male vocalization and female choice in the hybridogenetic *Rana lessonae*/*Rana esculenta* complex. *Anim. Behav.*, **60**: 745–755.

Rondinelli, B. 2006. Female choice in all-hybrid populations of *Rana esculenta*. MSc thesis, University of Zurich.

Rybacki, M. and Berger, L. 2001. Types of water frog populations (*Rana esculenta* complex) in Poland. *Mitt. Mus. Natkd. Berlin, Zool. Reihe*, **77**: 51–57.

Schultz, R.J. 1969. Hybridization, unisexuality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. *Am. Nat.*, **103**: 605–619.

Schwarzbach, A.E., Donovan, L.A. and Rieseberg, L.H. 2001. Transgressive character expression in a hybrid sunflower species. *Am. J. Bot.*, **88**: 270–277.

Seehausen, O. 2004. Hybridization and adaptive radiation. *Trends Ecol. Evol.*, **19**: 198–207.

Som, C. and Reyer, H.-U. 2006. Demography and evolution of pure hybridogenetic frog (*Rana esculenta*) populations. *Evol. Ecol. Res.*, **8**: 1235–1248.

Spaak, P. and Boersma, M. 2006. Predator mediated coexistence of hybrid and parental *Daphnia* taxa. *Arch. Hydrobiol.*, **167**: 55–76.

Turcotte, M.M., Pires, M.N., Vrijenhoek, R.C. and Reznik, D.N. 2008. Pre- and post-fertilization maternal provisioning in livebearing fish species and their hybrids (Poecilidae: Poeciliopsis). *Funct. Ecol.*, **22**: 1118–1124.

Voituron, Y., Joly, P., Eugene, M. and Barre, H. 2005. Freezing tolerance of the European water frogs: the good, the bad, and the ugly. *Am. J. Physiol.: Regul. Integr. Comp. Physiol.*, **288**: 1563–1570.

Vorburger, C. 2001. Fixation of deleterious mutations in clonal lineages: evidence from hybridogenetic frogs. *Evolution*, **55**: 2319–2332.

Vrijenhoek, R.C. 2006. Polyploid hybrids: multiple origins of a treefrog species. *Curr. Biol.*, **16**: 245–247.

Wissemann, V. 2007. Plant evolution by means of hybridization. *Syst. Biodivers.*, **5**: 243–253.

Zeisset, I., Rowe, G. and Beebee, T.J.C. 2000. Polymerase chain reaction primers for microsatellite loci in the north European water frogs *Rana ridibunda* and *R. lessonae. Mol. Ecol.*, **9**: 1173–1174.