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Correspondence

Cross-specific mortality and differential occurrence of aberrant phenotypes in tadpoles of the *Pelophylax* kl. *esculentus* hemiclone assortment

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Morphological malformations are one of the factors contributing to amphibian declines worldwide. There is growing evidence that these are boosted by altered ecological conditions like increased UV-B radiation, parasite infections, and pollution (e.g., Blaustein et al. 1997, Johnson et al. 2001). Recent advances in molecular techniques today facilitate more in-depth studies of inbreeding, i.e., the union of consanguineous gametes that can lead to a significant decrease in fitness by favouring the expression of recessive deleterious alleles in homozygotes (Keller & Waller 2002, Charlesworth & Willis 2009). Such reduction in genetic diversity can similarly promote the occurrence of morphological malformations in natural populations of many different taxa like fishes (Afonso et al. 2000), mammals (Mansfield & Land 2002), reptiles (Olsson et al. 1996), and amphibians (Williams et al. 2008).

Water frogs of the genus *Pelophylax* Fitzinger, 1843 (Anura, Ranidae) offer a unique opportunity for studying relationships between the onset of body malformations and inbreeding. Some species indeed exhibit a peculiar reproductive mode called hybridogenesis (Schultz 1969) in which two different parental species crossbreed to produce a fertile hybrid (hemiclone) that will carry the genomes of both parental species in its somatic cells and produce haploid gametes that only carry one non-recombinant parental genome. In eastern and central Europe, the *Pelophylax esculentus* complex (Frost et al. 2006) occurs, which involves two parental species: *Pelophylax lessonae* (Camerano, 1882) and *Pelophylax ridibundus* (Pallas, 1771), and their fertile hybrid *P.* klepton *esculentus* (Linnaeus, 1758) (Berger 1988). Prior to meiosis, *P.* kl. *esculentus* eliminates the *P. lessonae* genome (Borkin et al. 1979, Tunner & Heppich 1981), transmitting the *P. ridibundus* genome clonally. In northern Italy, *P.* kl. *esculentus* naturally coexists with *P. lessonae* in the so-called L-E system (Uzzel et al. 1976). Here, the fertile hybrid mates with the parental species to maintain the hybrid condition in each generation (Pagano et al. 1997), thus masking deleterious recessive alleles carried by the non-recombinant R genome via permanent heterozygosity with the host parental species (Berger 1967, Graf & Müller 1979, Graf & Polls-Pelaz 1989, Vorburger 2001).

As a result, natural mating between two hybrids in L-E systems typically leads to non-viable RR tadpoles (individuals that carry two clonally transmitted *ridibundus* genomes), as is evidenced by the absence of adult, and in most cases of metamorphosed juvenile *P. ridibundus* in L-E systems throughout their range, and the production of non-viable offspring, with high mortality rates during the early tadpole developmental stages (97%; Berger 1967, Graf & Polls-Pelaz 1989). Genomic interactions between different hemiclones instead lead to less negative outcomes (in terms of larval development and metamorphosis) according to the cross-specific expression of recessive deleterious mutations (Guex et al. 2002).

Here, we report the occurrence of a higher mortality rate and abnormal development, i.e., the incidence of an external morphological malformation in tadpoles generated by crossings between identical *P.* kl. *esculentus* same hemiclones from the same population in comparison with offspring resulting from crossbreeding between different hemiclones sampled from different populations.

Adult frogs were caught in the field from two different populations in northwestern Italy (Lombardy). Sampling sites were selected according to previous knowledge on the distribution of native taxa, to avoid collecting alien individuals or their hybrids (Bellati et al. 2012). Specifically, sampling was carried out at a lowland site (S1, Bernate Ticino: 45.47 N, 8.80 E, 120 m a.s.l.) and a highland site (S2, Armelio Mt.: 44.73 N, 9.45 E, 1025 m a.s.l.) during the peak of the reproductive season, which may last from late March through May depending on altitude; Lanza et al. 2007). Frogs were collected by hand or net using torchlights at night, and assigned to native taxa based on their

morphologies. In the field, specimens were measured with digital callipers (± 1.0 mm), and subadults (SVL, snout–vent length, < 60 mm; Lanza et al. 2007) were immediately released at their capture sites.

Adult frogs, two males (ABZC03417 from population S1 and ABZC03311 from population S2) and one female (ABZC03430 from population S1) were transferred to the laboratory and housed in plastic tanks filled with matured tap water, outfitted with bricks and leaves as shelters, external filters (EHEIM ecco pro 200, EHEIM, Germany), and a UV-B lamp (2.0%, Sera, Germany) each. The frogs were fed daily with live crickets (*Acheta domestica*) and meal beetles (*Tenebrio molitor*), fortified with a calcium and vitamin D_3 supplement (ZooMed, California, USA). Prior to their being accommodated thus, we toe-clipped the individuals to individualize them and collect biological samples for subsequent molecular analysis.

Genomic DNA was extracted using a commercial kit (Sigma-Aldrich, Saint Louis, USA) following manufacturer's instructions. Because members of this genus are difficult to identify morphologically and to confirm their phenotypic assignment, we firstly amplified the mitochondrial gene, encoding for NADH dehydrogenase subunit 3 (ND3, 340 base pairs in length), which discriminates between distinct *Pelophylax* taxa (Plötner et al. 2008). Amplifications were set up using 0.5 U of HotStart Taq DNA Polymerase (biotechrabbit GmbH, Hennigsdorf, Germany) and published primer pairs (Plötner et al. 2008). PCR products were sequenced externally (Eurofins Genomics, Ebersberg, Germany). Raw electropherograms were visually checked to exclude the presence of double peaks and translated into amino acids using Geneious v11 (Kearse et al. 2012) to detect possible premature stop codons suggesting the presence of pseudogenes. Aligned sequences were compared in GenBank (https://www.ncbi.nlm.nih.gov/) using the BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi) algorithm to infer mitochondrial species assignment.

As *P.* kl. *esculentus* carries a *lessonae*-type mtDNA in Italian populations of the L-E system, we also screened 9 codominant microsatellite markers that, according to the literature (Garner et al. 2000, Zeisset et al. 2000, Arioli 2007, Christiansen & Reyer 2009), are known to amplify only the *lessonae*-type genome (L-DNA), or only the *ridibundus*-type genome (R-DNA), or both. Prior to sequencing, the PCR products were combined in three mixes based on the mean lengths of alleles and dyes. Runs were performed externally (by Eurofins Genomics). Raw sequencing outputs were visually checked using Geneious to detect the presence genome-specific allele fragments. Allele dimensioning was performed with the same software.

According to our genetic analysis, the collected specimens were referable to the native L-E system, as ND3 sequences (Accession Numbers: MK124580-2) matched at 100% probability sequence AM749726 from northern Italy (*P. lessonae*, Italy: Carbonare; Plötner et al. 2008). Moreover, they were referable to *P.* kl. *esculentus* according to their nuclear genotypes, i.e., all loci were successfully amplified but only those assumed to be non-selective for the L- or R-genomes turned out to be heterozygotes. More precisely, genotypes from S1 (from male ABZC03417 and female ABZC03430) exhibited perfect allele-sharing at nuclear loci, attesting both individuals sharing the same hemiclone. In contrast, alleles of male ABZC03311 from population S2 were nearly entirely private, suggesting it was representative of a distinct hemiclone.

Breeder specimens were crossed in vitro following Berger's protocol (Berger et al. 1994) with minor modifications to optimise usage of clutches and sperm suspensions (Bellati et al. unpubl. data). Before manipulations, males were euthanised by immersion in a MS-222 water bath solution (0.5g/l). Mature eggs were obtained by gently pressing the female's venter.

Crossings were performed on 4 July 2017, and hatching occurred one week later, on 10 July 2017 (determined when 50% of the tadpoles had hatched from their eggs). A total of 150 viable tadpoles were selected from each crossing, divided into three replicates (50 tadpoles each), and raised indoors in plastic tanks filled with 10 litres of matured tap water under natural daylight conditions. Temperature was checked daily using a digital thermometer (± 0.1°C, Greisinger Electronic, Germany) to ensure homogeneity between tanks. Tadpoles were monitored daily to assess hatching dates and developmental stages according to Gosner (1960). After reaching stage 28, tadpoles were fed with dry rabbit food, and monitored for 80 days, which corresponds to the period required for native water frog tadpoles to reach metamorphosis according to literature (Lanza et al. 2007). Both malformation and death rates were recorded 42 days after hatching date (corresponding to Gosner stage 30 in our experimental offspring) and at the end of the observation period on 21 September 2017 (corresponding to Gosner stage 36). Under our standardized housing conditions, none of the individuals reached metamorphosis.

The level of mortality during the monitoring period was investigated using the two-sample Student's t-test. We observed a significantly higher mortality rate in tadpoles generated by the crossing between individuals from the same population (Crossing 1) than those by Crossing 2 in both observation trials (42 dd: $t = 13.868$, $p = < 0.05$; 84 dd: $t = 10.076$, $p = < 0.05$; Table 1). Much more interestingly, though, tadpoles generated by Crossing 1 (parents from the same population) showed a high malformation rate (bent tail, Fig. 1), whereas none of those generated by individuals from different populations (Crossing 2) exhibited tail malformations (Fig. 2).

The lower mortality rates in crossbreeds between different hemiclones of *P.* kl. *esculentus* sampled in geographically isolated populations suggests that the combination of sufficiently differentiated parental genomes may limit the expression of deleterious mutations in the RR offspring. This conclusion is in agreement with previous studies, in which, however, a positive effect of heterozygosity in RR offspring was reported only for crossbreeds between

Table 1. Mean mortality ± standard deviation of the crossings on Days 46 and 84 after hatching.

	Crossing 1	Crossing 2	t	p
mortality on d 46 ± SD	0.6 ± 0.00	0.27 ± 0.04	13.868	<0.05
mortality on d 84 ± SD	0.73 ± 0.05	0.35 ± 0.04	10.76	<0.05

deeply evolutionary differentiated lineages of *P.* kl. *esculentus* (from Switzerland and Sicily, respectively; Guex et al. 2002).

Figure 1. Ventral (top) and dorsal (below) views of a tadpole generated by crossing the same hemiclonal *P.* kl. *esculentus* from the same population (Crossing 1), showing the recurring bent tail malformation. Scale bars 5 mm.

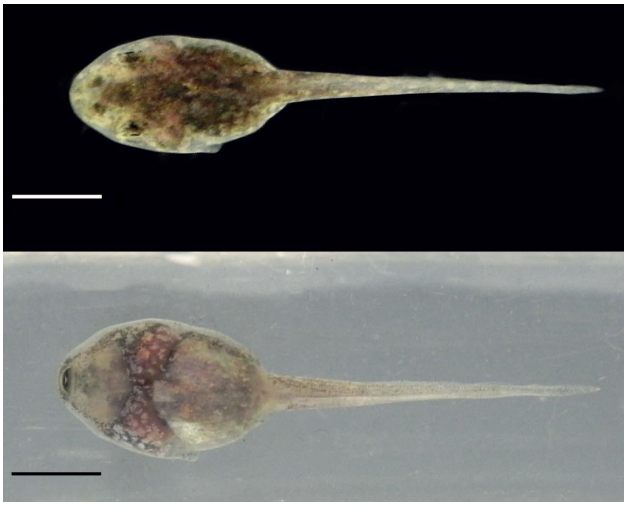

Figure 2. Ventral (top) and dorsal (below) views of a tadpole generated by crossing different hemiclones of *P.* kl. *esculentus* from distinct populations (Crossing 2), showing normal tail development. Scale bars 5 mm.

The main outcome of our experiment concerned the striking difference in the frequency of aberrant development of the tail in the cross between identical hemiclones (Crossing 1). Noteworthy here is that the same rate of aberrant individuals per tank (85% of the tadpoles) was recorded in each tank during the first trial. At that time, tadpoles were at Gosner's stage 30, i.e., they had just reached the free-swimming and foraging stage, so that the potential influence of environmental factors (mainly 'food' under our controlled conditions) could be considered negligible. This further supports the hypothesis that this particular malformation should be genetically based. Malformation rates changed slightly during the second trial (56, 77 and 81%, respectively) as a result of different mortalities in each tank.

Tail malformation can compromise the fitness of tadpoles in several ways. First of all, abnormal individuals suffer from greatly reduced swimming skills, resulting in obvious handicaps that will affect their feeding and capability of escaping threats. Moreover, it goes along with other malformations in the whole body plan and faulty organogenesis. Although our conclusions are drawn from the results of a single parental crossbreed each, the role of inbreeding in driving bent-tail malformation appears to be unquestionable, since the same female was mated with different males, excluding possible maternal effects on tadpole development between clutches. In addition, tadpoles were raised together under standardized housing conditions.

In conclusion, our results add knowledge to our understanding of the genetic basis of a phenotypically aberrant phenotype (bent tail) in tadpoles, and further support previous conclusions that inbreeding may be a strong causal factor that will determine mortality rates and the expression of developmental abnormalities in wild amphibian populations.

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