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Outbreeding depression in the common frog, *Rana temporaria*

Jörgen Sagvik[1,*], Tobias Uller[1,2] and Mats Olsson[1,2]
[1]*Department of Zoology, Göteborg University, Medicinaregatan 18, 405 30 Göteborg, Sweden;* [2]*Department of Biological Sciences, University of Wollongong, NSW 2522, Australia* *Corresponding author: *Phone: +46 (0) 31 773 36 32; Fax: +46 (0) 31 41 67 29; E-mail: jorgen.sagvik@zool.gu.se*

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Abstract

Theory suggests that parental relatedness is a continuous variable with a fitness optimum that we heretoforth will refer to as 'optimal outbreeding'. In the present paper, we test this proposition from a conservation (translocation) perspective. Amphibians are facing a global decline and many amphibian populations are today small and threatened by extinction. Because genetic differentiation is often high between amphibian populations, they could be particularly sensitive to outbreeding depression, e.g. due to breakdown of locally adapted gene complexes. We tested if outbreeding would reduce fitness in common frogs, *Rana temporaria,* crossed from a large and an isolated, small population, separated by 130 km, using artificial fertilization. For females from the large population, tadpoles were significantly smaller and more malformed in crosses with males from the small population, than with males from the large population. For the small population, however, no significant paternal genetic effects could be found. The difference in response to outbreeding between populations was accompanied with significant differences in the importance of maternal effects. We conclude that care should be taken when translocating frogs between distantly related populations to avoid outbreeding depression.

Introduction

One of the central questions in evolutionary biology is whether species consist of relatively small, subdivided populations with restricted gene flow, or large homogeneous ones (e.g. Hanski & Simberloff 1997). If populations are subdivided, different selection pressures are expected to lead to local adaptation. With the additional effect of genetic drift, populations will diverge genetically, ultimately leading to speciation.

Genetic divergence can be found on the scale of meters (Waser & Price 1989; Hitchings & Beebee 1997, Edmands 1999), up to several thousand kilometres (Edmands 1999), depending on the species and environmental factors. Amphibians show the strongest genetic differentiation between populations of all vertebrates (Ward et al. 1992; Driscoll 1998). This is partly due to restricted dispersal abilities (e.g. Berven & Grudzien 1990; Ward et al. 1992; Driscoll 1998, but see Seppä & Laurila 1999; Newman & Squire 2001), strong philopatry (Berven & Grudzien 1990; Reading et al. 1991), and a patchy distribution of breeding localities (Ward et al. 1992; Driscoll 1998; Lampert et al. 2003). Furthermore, amphibian species are commonly composed of a mosaic of large and small populations, differing in degree of genetic variation and often with very restricted gene flow even between closely situated populations (Hitchings & Beebee 1997; Newman & Squire 2001; Lampert et al. 2003; Brede & Beebee 2004). Consequently, amphibians should be good model organisms for studies of local adaptation and the causes and consequences of inbreeding and outbreeding. The strong subdivision of populations and resulting inbreeding, often due to habitat fragmentation as a result of human activities,

could also contribute to global amphibian decline (Wake 1991; reviewed by Alford & Richards 1999).

Theory suggests an optimal genetic distance (often correlated with geographic separation) between parents when offspring fitness is maximized (Bateson 1982; Waser & Price 1989; Keane 1990). Inbreeding, i.e. matings between closely related individuals, is detrimental mainly because of expression of deleterious recessive alleles (Ledig 1986; Keller & Waller 2002), but also because it results in low genetic diversity, e.g. at the MHC complex (Madsen et al. 1999). Outbreeding, on the other hand, could lead to genetic parental incompatibility, and result in breakdown of co-adapted gene complexes, or production of intermediate phenotypes with less perfect local adaptation (Alstad & Edmunds 1983; Templeton 1986; Waser & Price 1989; Shields 1993). The effects of in- and outbreeding are also likely to depend on the population of origin, for the following reasons. First, a long history of inbreeding can (but not necessarily will) lead to purging of deleterious alleles (Keller & Waller 2002). Thus, further inbreeding need not be detrimental to fitness, although the population may show low genetic variation. Outbreeding depression, on the other hand, is likely because of disruption of co-adapted gene complexes in crossings between individuals from small populations (Templeton 1986). Furthermore, with restricted gene flow, populations under selection will adapt to local conditions. Thus, outbreeding should more often lead to detrimental effects in crosses between populations that differ in local adaptive optima. Therefore, optimal outbreeding (i.e. highest fitness) should differ among isolated populations depending on their sizes, with small populations having more benefit of outbreeding than large populations with already high genetic variation (but see Templeton 1986). Finally, if small populations are purged of their genetic load, introduction of individuals from such populations could lead to less detrimental effects than introduction of animals from larger populations with higher genetic load (Amos & Balmford 2001). However, new mildly deleterious mutations will accumulate with time (Bataillon & Kirkpatrick 2000). Furthermore, whether or not purging is likely to occur in natural populations is debated (Keller & Waller 2002). In the present paper, we investigate the consequences of outbreeding by comparing fertilization success, malformation frequency, and hatchling size of *Rana temporaria* tadpoles in crosses between two populations differing in population size and isolation.

Materials and methods

The common frog (*Rana temporaria*) is a medium sized Ranid frog (total length of about 8–9 cm), distributed over large parts of Europe. Studies of genetic differentiation between populations have found differences at distances as small as 2.3 km (Hitchings & Beebee 1997). Many studies have also found strong evidence for local adaptation to environmental conditions (e.g. Laurila et al. 2001; Ståhlberg et al. 2001; Laugen et al. 2002; Loman 2003) even over short geographic distances (Loman 2003, see also Räsänen et al. 2003 for another Rana species).

Forty-four frogs (22 females, 22 males) from Onsala (57°26′N, 11°59′E) and 44 (22 females, 22 males) from Dingle (58°31′N, 11°34′E), in southwestern Sweden, were captured within a few days in April 2003. The Onsala population is large with an estimated (based on number of clutches) size of more than 2000 breeding pairs (henceforth referred to as the ''large population''), while Dingle is a small population with approximately 50–75 breeding pairs; referred to as the ''small population''. The small population is isolated with no closely situated breeding ponds (>5 km), and therefore has very restricted migration contributing to genetic exchange (T. Helin, personal communication). The two populations are separated by approximately 130 km, i.e. there is no direct gene flow between them. The frogs were transported to the laboratory at Göteborg University and kept in 4 °C for 5–9 days before the onset of the experiment. Individual mass of each frog was measured to the nearest 0.01 g on an electronic scale. The artificial fertilization procedure followed the protocol outlined by Berger et al. (1994). The frogs were injected with hormones (LHRH, Sigma-Aldrich), which induces ovulation within 24 h while males shed their sperm into the cloaca within 1 h of the injection. Eggs were gently stripped from each female by squeezing her abdomen and partitioned into two Petri dishes, each containing a sperm solution obtained from one randomly chosen male, from either the small

or the large population. Thus, eggs from each female were fertilized by one male from the same population, and one male from the other population. Each male was used twice in one within-population, and one between-population cross.

The sperm/egg mixture was immediately covered with tap water (Göteborg, Sweden), which was continuously replenished when absorbed by the eggs. One of the males did not release sperm into his semen and his crosses were therefore excluded from the experiment. Approximately 20 eggs from each female were put in 5% formaldehyde for later analysis of maternal investment (i.e. egg size).

Experimental design

A pool system was arranged with two replicate pools (152 × 122 × 25 cm, henceforth A and B) per two water temperature treatments (15 and 20 °C). Eighty-six 1-L plastic jars with wire mesh bottoms (to ensure water circulation) were hung from crossbars in all four pools (see Ståhlberg et a1. 2001, for a similar design). The four pools were set up using tap water (Göteborg, Sweden) that was aerated for a minimum of 10 days before the onset of the experiments. A water conditioner (Aquatan, Heisenberg, Germany) was used to eliminate any potential heavy metal ions in the water. The ambient temperature was adjusted to keep the water temperature in the pools to 15 °C. Two pools were then heated to 20 °C using submersible, commercially available aquarium heaters (Jäger, Germany). The water temperatures fluctuated no more than ±1 °C throughout the experiment. Approximately 60 eggs were put in each jar, i.e. ca. 240 eggs per cross were used.

When ca. 95% of the eggs had hatched (stage 23; Gosner 1960), the tadpoles were removed and put in 5% formaldehyde, for subsequent. measuring and scoring of malformations. Completely undeveloped eggs were classified as infertile. There was virtually no incidence of embryonic death before hatching, and fertilization success was therefore calculated as the proportion of hatched eggs (or nearly hatched, i.e. tadpoles that were close to hatching at sampling). Four randomly chosen tadpoles from each replicate were measured (total length, tail length, length from snout to gill and tail width) in a stereoscope to the nearest 0.06 mm (set by the distance between ruler bars in the eye-piece of the stereoscope). All tadpoles were inspected for signs of severe malformations such as kinked vertebrae, enlarged abdomen, malformed body or tail, but no effort was made to qualitatively separate malformations. To obtain a measure of maternal investment, two measurements of egg diameter from each of 12 eggs per female were taken to the nearest 0.06 mm using a stereoscope. Statistical analyses were performed in SAS 8.2.

Results

Males and females from the small population were significantly larger than those from the large population (ANOVA; males: $F_{1,38} = 38.0$, $P < 0.001$; females: $F_{1,41} = 42.2$, $P < 0.001$). Egg size did not show any difference between populations (ANOVA; $F_{1,41} = 0.04$, $P = 0.83$), but when controlling for female size using female mass as a covariate, females from the large population had relatively larger body size-specific egg size (ANCOVA; female population: $F_{1,40} = 14.7$, $P < 0.001$; female mass: $F_{1,41} = 26.1$, $P < 0.001$). In our analysis of hatchling traits, we first verified that there was no difference between replicates for any of the traits examined ($P > 0.90$), and we therefore pooled the replicates for further analysis. When comparing the two populations (i.e. using pure crosses only), there was a higher, although non-significant percentage of malformations in the small population (logistic regression; temperature: $\chi^2_{1,77} = 0.82$, $P = 0.37$; population: $\chi^2_{1,77} = 3.43$, $P = 0.064$; temperature × population: $\chi^2_{1,77} = 0.33$, $P = 0.56$), and significantly smaller hatchling size (ANCOVA, controlling for egg size; temperature: $F_{1,76} = 1.31$, $P = 0.26$; population: $F_{1,76} = 4.07$, $P = 0.047$; temperature × population: $F_{1,76} = 2.95$, $P = 0.090$).

Because the same pairs of males were used in crosses with females from both populations, we analysed our data separately for females from the large and the small population, using an additive repeated measures design with females treated as random blocks (Quinn & Keough 2002).

Fertilization success did not differ between within versus between population crosses for either population (logistic regression: small population: female: $\chi^2_{21,19} = 27.8$, $P = 0.15$, male population: $\chi^2_{1,19} = 0.02$, $P = 0.89$; large population:

Table 1. Results from logistic regressions with presence of malformations as response variable for crosses of females from the large and the small population.

Source	$Female_{large\ population}$			$Female_{small\ population}$		
	χ^2	*P*	N	χ^2	*P*	N
Female	70.2	< 0.001	20,55	222.4	< 0.001	21,59
Male population	6.78	0.009	1,55	1.50	0.22	1,59
Temperature	3.53	0.060	1,55	3.51	0.061	1,59
Male population × temperature	0.00	0.94	1,55	1.41	0.23	1,59

female: $\chi^2_{20,17} = 40.6$, $P = 0.004$, male population: $\chi^2_{1,17} = 1.96$, $P = 0.16$), with an overall fertilisation success of 70%. For females from the large population, outbreeding resulted in significantly higher incidence of malformed hatchlings than crosses with a male from the same population (Table 1, Figure 1). For the small population, there was a similar, but non-significant pattern of increased incidence of malformations under outbreeding (Table 1, Figure 1). Furthermore, for the large population, both temperature and male population identity had a significant effect on hatchling size, whereas for the small population none of the factors, except for female identity, were significant (Table 2, Figure 2). Hatchlings from large population females were larger in 15 °C. Unfortunately, egg size cannot be incorporated into the present analyses, because egg size and female identity are indistinguishable. However, it is worth noting that egg size was not correlated with hatchling size for females from the large population (Pearson's partial correlation, controlled for female size; means per female; $r_p = -0.10$, $P = 0.68$; only pure crosses: $r_p = -0.30$, $P = 0.23$, Fig. 3a), whereas in the small population, egg and hatchling size were strongly correlated (means per female; $r_p = 0.61$, $P = 0.003$; only pure crosses: $r_p = 0.71$, $P < 0.001$, Fig 3b). The relationship between egg size and hatchling size therefore differed significantly between populations (heterogeneity of slopes test; $F_{1,37} = 11.6$, $P = 0.030$; only pure crosses: $F_{1,37} = 11.6$, $P = 0.002$).

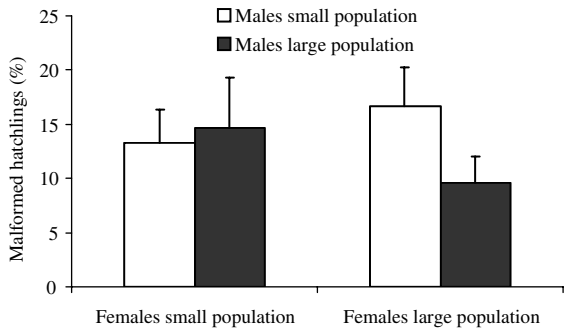

Figure 1. Percentage malformed hatchlings from different crosses (mean values ± SE). See Table 1 for test statistics.

Discussion

When populations are geographically separated with restricted gene flow, local selection pressures and genetic drift will lead to among-population genetic differentiation. In amphibians this process seems exceptionally fast, resulting in the highest genetic divergence recorded among vertebrate populations (Ward et al. 1992). Our results from artificial crosses between individuals of the common frog, *Rana temporaria,* from two populations, one isolated and small, and one large (approximately 50–75 *versus* 2000 breeding pairs), separated by 130 km, suggest genetic differentiation between the two populations, which can explain our demonstration of population-specific outbreeding depression. Offspring of females from the large population showed higher incidence of malformations and smaller hatchling size when fathered by males from the small population. However, there was no significant effect of paternal genes on hatchling traits in matings with females from the small population. The lack of response to outbreeding in the small population could potentially be due to the strong maternal effects in the small, but not the large, population.

Table 2. Results from ANOVAs with hatchling length as response variable for crosses of females from the large and the small population.

Source	$Female_{large\ population}$			$Female_{small\ population}$		
	F	*P*	d.f.	F	P	d.f.
Female	2.84	< 0.001	20,55	10.65	< 0.001	21,59
Male population	7.26	0.009	1,55	1.70	0.19	1,59
Temperature	8.06	0.006	1,55	0.57	0.45	1,59
Male population × temperature	0.10	0.76	1,55	2.38	0.13	1,59

Our analyses of pure crosses, i.e. comparing the two unmanipulated populations, showed smaller hatchling size (controlled for egg size), and a borderline significant higher incidence of malformations in the small compared with the large population. Although the smaller hatchling size could be explained by genetic differences between populations, it could also be a consequence of detrimental effects due to inbreeding, which is likely to be the reason for the (marginally significant) higher incidence of malformations. Thus, from a conservation perspective, translocating individuals from a larger population could be used as a means to avoid inbreeding in this species. However, females from the large population suffered negative effects of male genetic contributions from the small population, with a higher incidence of malformed hatchlings under outbreeding, even higher than the degree of malformations in the "pure" inbred matings. Thus, this runs counter to the argument that small populations are purged of detrimental recessives. Furthermore, eggs from the large population developed into smaller hatchlings when fertilized by males from the small population. If this was the result of parental genetic effects only, we would have expected similar effects in the reciprocal crossings in both populations. This was not the case, and the direction of paternal effects based on male size for females from the small population was even opposite to that predicted based on pure genetic differentiation between populations (Figure. 2). Thus, from a female's perspective, outbreeding generates negative effects for the large, but not the small population, both with respect to offspring quality (as determined by incidence of malformations), and hatchling size, which is likely to be related to size

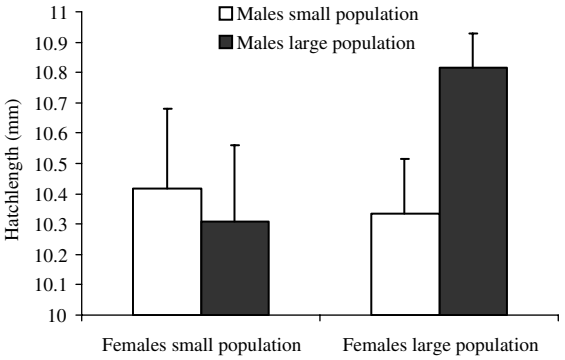

Figure 2. Length of hatchlings from different crosses (mean values ± SE). See Table 2 for test statistics.

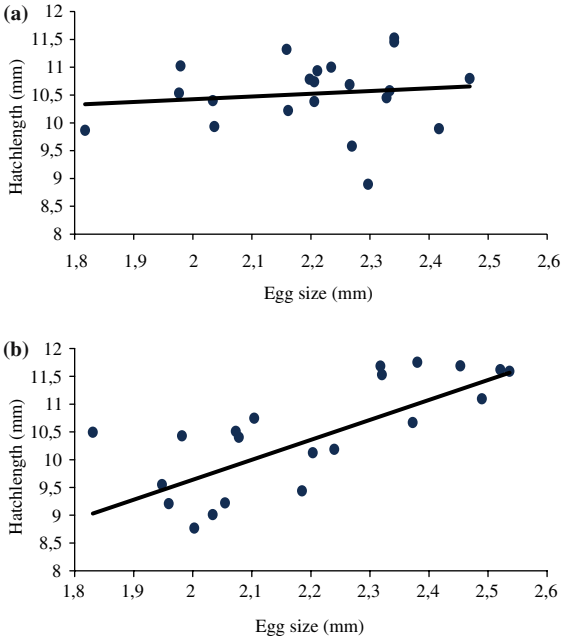

Figure 3. Relationship between egg size and hatchling length for (a) the large, and (b) the small population.

at metamorphosis, and ultimately fitness (Altwegg & Reyer 2003).

Outbreeding depression has been found in relatively few natural animal populations, especially in intraspecific matings (Alstad & Edmunds 1983; Brown 1991; Edmands 1999; Marr et al. 2002), but could be common in taxa such as amphibians that often show strong genetic differentiation between populations (Hitchings & Beebee 1997; Newman & Squire 2001; Lampert et al. 2003; Brede & Beebee 2004). The current, worldwide decline of amphibians has led conservationists to propose translocation of frogs from large (outbred) populations to isolated, declining populations, to increase their genetic variation (Reinert 1991; Seigel & Dodd 2002; Trenham & Marsh 2002). As indicated by the present study, such introductions could potentially lead to reduced fitness, even if the populations are not separated by more than some hundred kilometres (and may be much less, Hitchings & Beebee 1997). Future work should determine whether the pattern found in the present study can be repeated with other populations differing in size and geographic/genetic divergence.

Nevertheless we suggest that care should be taken when introducing new genetic material to save threatened amphibian populations. The procedure outlined in the current paper offers a technique with which potential outbreeding effects can be assessed before translocation takes place.

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