A genetic component of resistance to fungal infection in frog embryos

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The embryo has traditionally been considered to completely rely upon parental strategies to prevent threats to survival posed by predators and pathogens, such as fungi. However, recent evidence suggests that embryos may have hitherto neglected abilities to counter pathogens. Using artificial fertilization, we show that among-family variation in the number of *Saprolegnia*-infected eggs and embryos in the moor frog, *Rana arvalis*, cannot be explained by maternal effects. However, analysed as a within-females effect, sire identity had an effect on the degree of infection. Furthermore, relatively more eggs and embryos were infected when eggs were fertilized by sperm from the same, compared with a different, population. These effects were independent of variation in fertilization success. Thus, there is likely to be a significant genetic component in embryonic resistance to fungal infection in frog embryos. Early developmental stages may show more diverse defences against pathogens than has previously been acknowledged.

Keywords: fungal infection; maternal effects; pathogen; *Rana arvalis*; *Saprolegnia*

1. INTRODUCTION

Fungal infection during embryonic development has recently been identified as a potentially strong selective agent in ectotherm egg-layers (Kiesecker & Blaustein 1995; Green 1999; Gomez-Mestre *et al.* 2006). In free-spawning amphibians, eggs are commonly oviposited all at once, are unattended by females and, consequently, are subject to the risk of infection. This should favour the evolution of maternal strategies (e.g. allocation of immunological factors in relation to perceived risk of infection; Grindstaff *et al.* 2003) that limit or prevent fungal infection of developing embryos (Hamdoun & Epel 2007), suggesting that variation among families in susceptibility to fungal pathogens may be due to maternal effects (Sagvik *et al.* 2008). However, variation in the degree of infection could also reflect genetic variation in embryo-specific immunity or offspring developmental plasticity in response to external threats to survival that do not rely upon maternal transfer of resources or information (Hamdoun & Epel 2007). For example, in the wood frog (*Rana sylvatica*), the American toad (*Bufo americanus*) and the spotted salamander (*Ambystoma maculatum*), fungal infection by *Saprolegnia* induces premature hatching to different degrees and, hence, escapes from a potential severe threat to survival (Gomez-Mestre *et al.* 2006; Touchon *et al.* 2006).

We have previously documented significant among-family (clutch) variation in the degree of *Saprolegnia* infection of eggs and embryos in the moor frog, *Rana arvalis*, following experimental exposure to the fungus (Sagvik *et al.* 2008). However, there was no evidence for developmental plasticity (assessed as time to, and size at, hatching) and we therefore suggested that the

among-family variation was due to variation among mothers in some aspect of allocation to eggs or the surrounding jelly coat. In the present study, we use a half-sib design to test this proposition against the alternative hypothesis of genetic variation in embryonic resistance.

2. MATERIAL AND METHODS

The moor frog, *R. arvalis*, is a common lowland frog species with a wide distribution in Eurasia. The fungal pathogen *Saprolegnia* commonly infects moor frog eggs with severe consequences for embryonic survival in some populations and years (van Gelder & Wijnands 1987; J. Loman 2005, personal communication). Previous studies of the present species have shown variation in fungal infection at the levels of family, population and thermal conditions (Sagvik *et al.* 2008).

From each of four localities in southwestern Sweden (Björkö 57°44' N, 11°40' E; Öckerö 57°43' N, 11°38' E; Måryd 55°42' N, 13°21' E and Frihult 55°33' N, 13°38' E), 7 female and 14 male frogs were captured at the onset of the breeding season 2005 and transported to the laboratory at the University of Gothenburg where they were kept in the dark (for keeping the animals inactive) at 4°C for a maximum of 10 days before the onset of the experiment. The artificial fertilization procedure followed the protocol outlined by Berger *et al.* (1994). Sperm was obtained by injecting males with luteinizing hormone-releasing hormone (LHRH). After female hormonal manipulation, eggs were stripped from each female into two Petri dishes. Each Petri dish contained a sperm solution obtained from a randomly chosen male; one from the same population and one from a different population. Each male was used in only one cross. After 5–10 min, the sperm/egg mixture was covered with aerated tap water (Göteborg, Sweden). After approximately 2 hours, the eggs from each Petri dish were separated into four evenly sized egg masses and placed in four plastic jars, for a total of eight jars per female (mean $\pm$ s.e., number of eggs 68.1 ± 2.85 , $N = 221$; three control jars had missing values). Each jar

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was filled with 0.95 l of aerated tap water and kept at 18 ± 0.2 s.e. °C and a 14 : 10 L : D regime.

The pathogenic fungus *Saprolegnia* spp. (identified by Prof. N. Hallergren, Department of Botany, University of Gothenburg) was collected from the wild, but from another population than the ones used in this study (Onsala 57°26' N, 11°59' E). A small sample of fungus was cultured on agar (half strength Difco Emerson YpSs Agar) as per instructions by the manufacturer. A second pure culture was then topped with boiled hemp seeds and placed in 25°C for 3 days for the standardized sampling of *Saprolegnia* (Robinson *et al.* 2003; Sagvik *et al.* (2008) for a similar approach). Of the four jars per cross, the eggs in two jars were infected with *Saprolegnia* and the other two were kept as controls. One *Saprolegnia*-infected or non-infected (control) hemp seed (vector) was placed on the eggs when they had reached stage 15 ± 1 (approx. 2 days at 18°C; Gosner 1960). The hemp seed was placed centrally on top of the fertilized eggs and was in direct contact with the egg jelly continuously. Jars were inspected twice daily for hatchlings. At hatching (approx. 95% of developing embryos reached stage 23 in 9–11 days; Gosner 1960), all tadpoles, embryos and undeveloped eggs were counted and scored for *Saprolegnia* infection (infected or not infected). Undeveloped eggs were classified as *Saprolegnia* infected if they showed any signs of *Saprolegnia* growth, or otherwise as 'unfertilized'. This classification did not allow us to discriminate between infertile eggs infected by *Saprolegnia* and viable eggs killed by early *Saprolegnia* infection. However, by assessing proportion of infertile eggs in control jars for each cross, we could control for cross-specific fertility success in our statistical analysis. All data are presented as means $\pm$ s.e.

3. STATISTICAL ANALYSES

Because *Saprolegnia* infection only occurred in experimental jars, data from control jars were only used to obtain an unbiased estimate of the fertilization success of each sire. Ideally, the effect of male and female identity on the incidence of infected eggs and embryos should be estimated using a generalized linear mixed model (GLMM). However, because this approach forced us to rely on pseudo-likelihood estimates, no statistical test of random effects was possible. We therefore proceeded in two steps. First, we used GLMM (PROC GLIMMIX in SAS–STAT v. 9.1; see Littell *et al.* (2006) for details) with a Poisson distribution and a log link function to estimate the effects of female and male identities (nested within female identity) on the number of infected eggs and embryos. The analysis was run both with and without the proportion of fertile eggs from control jars as a covariate. Because female identity had approximately zero variance estimate in both these analyses, it was dropped from further analyses. The second step was therefore to run models with male and female population of origin and male identity (nested within male origin) as factors. The results from the GLMM were compared with those from a linear mixed model (LMM, PROC MIXED in SAS–STAT v. 9.1, i.e. assuming a Gaussian distribution) to assess whether the violation of the distribution assumption affected tests of fixed factors and to provide approximate statistical tests of random effects. Finally, the effect of male identity and male population was also analysed using non-parametric analysis of variance (Kruskal–Wallis test) and

paired Wilcoxon signed-rank test to ensure that the results were robust.

Throughout the analyses, we used the number of infected eggs and embryos per jar as our response variable rather than proportions since there was no correlation between the number of infected eggs and embryos and the total number of eggs per cross ($r=0.15$, $p=0.13$; infection rates were low (5%) compared with the total number of eggs) and because this response variable less strongly violated assumptions of a Gaussian distribution of the data (but deviated significantly from normality, $W=0.79$, $p<0.001$) and, hence, provide relatively better estimates for LMM. We report the covariance parameter estimates for random effects and associated statistical tests whenever available. Degrees of freedom for fixed effects were estimated using Satterthwaite's approximation (Littell *et al.* 2006).

4. RESULTS

Time to hatching showed non-detectable variation among jars within females and virtually all clutches hatched during the same day. Thus, we did not have sufficient resolution to test for variation in embryonic duration potentially arising from the *Saprolegnia* treatment. An initial model (PROC GLIMMIX) of the number of infected eggs and embryos showed that the covariance parameter estimate for female identity was approximately zero, whereas male identity had a variance component of 0.83 ± 0.211 (PROC MIXED: male identity = 5.86 ± 1.80 , $\chi^2=14.9$, $p<0.001$). These results were very similar when we included the proportion of fertile eggs (i.e. fertilization success) in the control jars as a covariate in the model, which was negatively correlated with the number of infected eggs and embryos (PROC GLIMMIX: proportion of fertilized eggs: $F_{1,41.6}=6.08$, $p=0.018$, female identity ≈ 0 , male identity = 0.74 ± 0.196 ; PROC MIXED: proportion of fertilized eggs: $F_{1,54.3}=9.25$, $p=0.004$, female identity ≈ 0 , male identity = 4.68 ± 1.609 , $\chi^2=11.2$, $p<0.001$).

We therefore dropped female identity and ran a new model with male population of origin, female population of origin and their interaction as fixed factors; fertilization success as a covariate; and male identity nested within origin as a random factor. The result showed that there was a significant interaction between male and female population of origin and suggested that male identity explained a significant proportion of variation in the number of infected eggs and embryos (table 1; figure 1). In general, the degree of infection was higher when eggs were fertilized by sperm from the same, than from a different, population (figure 1). Finally, a Kruskal–Wallis test supported the heterogeneity in the number of infected eggs and embryos among males ($\chi^2_{56}=75.7$, $p=0.041$) and a paired Wilcoxon signed-rank test confirmed the effect of between population crosses, with more eggs and embryos being infected in within-population crosses (within versus between populations, 4.2 ± 0.66 versus 1.52 ± 0.31 ; paired test within females, $p=0.002$; figure 1). In summary, our analyses support the effect of male identity by (i) covariance parameter estimates using GLMM, (ii) statistical tests from LMM, and (iii) non-parametric statistics.

Table 1. (a). Generalized linear mixed model (PROC GLIMMIX, SAS v. 9.1) with number of infected eggs and embryos as response variable. Note that no statistical test for male identity is provided as its estimate is based upon pseudo-likelihood procedures. (b). Linear mixed model (PROC MIXED, SAS v. 9.1) with number of infected eggs and embryos as response variable. (Pop, population of origin; * $p \leq 0.05$, ** $p \leq 0.01$.)

	(a)			(b)		
	estimate	χ^2		estimate	χ^2	
random effects						
male identity (male pop)	0.53 ± 0.167	—		2.85 ± 1.398	5.1*	
fixed effects	n.d.f.	d.d.f.	F	n.d.f.	d.d.f.	F
female pop	2	48.3	0.48	2	47	1.40
male pop	2	48.0	3.79*	2	47	4.00*
female pop $\times$ male pop	2	48.2	5.93**	2	46.9	5.89**
fertilization success	1	32.6	4.04*	1	45.9	6.95**

Figure 1. Mean number of infected eggs and embryos from within and between population crosses for females from four different populations (open bars, within population; filled bars, between population). On average, between-population crosses resulted in fewer infected eggs and embryos than did within-population crosses (see text for test statistics).

5. DISCUSSION

Families (clutches) vary in their degree of infection to *Saprolegnia* in both toads and frogs (Gomez-Mestre *et al.* 2006; Sagvik *et al.* 2008). Our present results suggest that, in *R. arvalis*, maternal effects do not represent a major contribution to variation in infection of eggs and embryos as has previously been hypothesized (Sagvik *et al.* 2008). Our artificial fertilization experiments instead suggest that the degree of infection of eggs and embryos was dependent on the identity of the sire and, hence, that there is an embryonic (diploid) genetic component of resistance to fungal infection. Furthermore, our results showed a higher incidence of infection when females were mated with a male from their own, than from a different, population. Thus, these results corroborate our previous findings of family and population variation in susceptibility to fungal growth and suggest that the main source of family variation is to be found in genetic variation in embryonic traits rather than maternal effects. Thus, there may be specific adaptations to avoid fungal infection in frog embryos with standing genetic variation for these traits in our populations. The decreased infection risk when eggs were fertilized by sperm from a male of a different population further suggests the presence of epistatic interactions. However, detailed dissection of these effects would require a more extensive quantitative genetic design.

Recent studies of amphibians have suggested that a proximate cause of variation in fungal infection could be induced hatching (Gomez-Mestre *et al.* 2006; Touchon *et al.* 2006). Although it cannot be completely discarded due to low levels of infection per jar, the low variation in time to hatching suggests that variation in the duration of embryonic development or developmental plasticity of growth rates under *Saprolegnia* infection is unlikely to explain our results (Sagvik *et al.* 2008). Thus, our data may instead suggest the presence of embryo-specific immunity (Hamdoun & Epel 2007). The nature of this proposed immune function is unknown but an active role of the developing embryo in preventing (rather than just escaping) fungal infection is supported by the observation that infertile eggs or dead embryos are more likely to be infected than live embryos (Kudo & Teshima 1991; Robinson *et al.* 2003) and by an increased number of infected eggs from clutches with a higher degree of infertility, also in species with limited plasticity in developmental timing (Sagvik *et al.* 2008; present study). Thus, females mated to males with low fertilizing ability may pay a twofold cost—in terms of both the number of offspring and an increased risk for the fertilized eggs to be infected by fungi (Moreira & Barata 2005), in particular under conditions generally promoting infection (e.g. low temperature and early exposure to the fungus, Kiesecker & Blaustein 1995, Robinson *et al.* 2003 and Sagvik *et al.* 2008). An intriguing, but highly speculative, alternative to genetic variation in embryonic defence is that sperm differ in their effect on the cortical reaction and, hence, on the transformation of the vitelline envelope to the fertilization envelope, as the latter has been shown to be responsible for the fungicidal effect in fish eggs and embryos (via enzymes released at the time of fertilization, Kudo & Teshima 1991). Combining artificial fertilization with studies of pathogen-induced mortality under field conditions (e.g. by transferring laboratory-produced clutches to natural ponds) is a next important step to investigate how important environmental conditions and the level of genetic variation in embryonic defences are in the wild. Understanding the nature and relative contribution of maternal and embryonic mechanisms, and strategies to reduce mortality arising from pathogens and predators, will be a stimulating future challenge for evolutionary ecologists, and of potential applied value (Hamdoun & Epel 2007).

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