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Differences in susceptibility to *Saprolegnia* infections among embryonic stages of two anuran species

María José Fernández-Benéitez · Manuel Eloy Ortiz-Santaliestra · Miguel Lizana · Javier Diéguez-Uribeondo

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Abstract Many amphibians are known to suffer embryonic die-offs as a consequence of *Saprolegnia* infections; however, little is known about the action mechanisms of *Saprolegnia* and the host–pathogen relationships. In this study, we have isolated and characterized the species of *Saprolegnia* responsible for infections of embryos of natterjack toad (*Bufo calamita*) and Western spadefoot toad (*Pelobates cultripes*) in mountainous areas of Central Spain. We also assessed the influence of the developmental stage within the embryonic period on the susceptibility to the *Saprolegnia* species identified. Only one strain of *Saprolegnia* was isolated from *B. calamita* and identified as *S. diclina*. For *P. cultripes*, both *S. diclina* and *S. ferax* were identified. Healthy embryos of both amphibian species suffered increased mortality rates when exposed to the *Saprolegnia* strains isolated from individuals of the same population. Embryonic developmental stage was crucial in determining the sensitivity of embryos to *Saprolegnia* infection. The mortalities of *P. cultripes* and *B. calamita* embryos exposed at Gosner stages 15 (rotation) and 19 (heart beating) were almost total 72 h after challenge with *Saprolegnia*, while those exposed at stage 12 (late gastrula) showed no significant effects at that time. This is the first study to demonstrate the role of embryonic development on the sensitivity of amphibians to *Saprolegnia*.

Keywords Amphibians · Oomycetes · Water moulds · Emergent diseases · Age-related sensitivity

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M. J. Fernández-Benéitez (✉) · M. Lizana
Departamento de Biología Animal, Universidad de Salamanca, Campus Miguel de Unamuno, 37007 Salamanca, Spain
e-mail: maybeneitez@usal.es

M. E. Ortiz-Santaliestra
Instituto de Investigación en Recursos Cinegéticos UCLM-CSIC-JCCM, Rda de Toledo s/n, 13071 Ciudad Real, Spain

J. Diéguez-Uribeondo
Departamento de Micología, Real Jardín Botánico CSIC, Plaza Murillo 2, 28014 Madrid, Spain

Introduction

Emergent diseases are among the main causes involved in amphibian declines. Bacteria, viruses and parasites cause individual mortality and affect the population status of many amphibian species (Densmore and Green 2007). Fungal infections are also involved in these declines; for example, chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis*, has been identified as a causal agent of the decline and extinction of amphibian populations in some locations (Daszak 1998; Daszak et al. 2003; Bosch et al. 2001; Rachowicz et al. 2005), and it can disrupt ecological interactions among amphibian species (Johnson et al. 2007).

Another common disease is the so-called *Saprolegnia* infection, which is caused by at least two species of *Saprolegnia*: *S. ferax* (Blaustein et al. 1994; Kiesecker et al. 2001) and *S. diclina* (Fernández-Benéitez et al. 2008). *Saprolegnia* belongs to the oomycetes, which are heterokont organisms that are phylogenetically unrelated to true fungi. Oomycetes produce swimming zoospores—which are important for their dispersion in aquatic environments—and are often considered the primary units of infection in many parasitic species (Diéguez-Uribeondo et al. 1994). These zoospores are biflagellate and quickly encyst to form primary cysts that can germinate or release a new generation of zoospores (Diéguez-Uribeondo et al.

1994, 2007; Fernández-Benéitez et al. 2008; Ke et al. 2009; Ghiasi et al. 2010). This ability may increase the likelihood of finding an appropriate substratum to germinate and thus ensuring survival (Bangyeekhun et al. 2003).

The genus *Saprolegnia* includes pathogens of freshwater animals and their eggs, and some of them are responsible for economically important diseases affecting farmed and wild populations of fishes (Willoughby 1978; Lategan et al. 2004; Zaror et al. 2004; van West 2006). The negative effects of *Saprolegnia* on aquatic stages of several amphibian species have been demonstrated (Blaustein et al. 1994; Kiesecker et al. 2001; Fernández-Benéitez et al. 2008; Sagvik et al. 2008a,b; Romansic et al. 2009; Ruthig 2009), and have been associated with the extinction of populations of *Rana pipiens* and *Bufo terrestris* (Bragg 1958, 1962), increased mortality in salamander *Ambystoma maculatum* (Walls and Jaeger 1987), and massive deaths of *B. calamita*, *R. temporaria* (Banks and Beebee 1988; Beattie et al. 1991) and *B. boreas* (Blaustein et al. 1994) eggs.

However, little is known about the action mechanisms of pathogenic *Saprolegnia* spp. and their host–pathogen relationships. Host susceptibility to infection may vary with development, and thus embryos of *B. boreas* and *Pseudacris regilla* are known to be sensitive to *Saprolegnia* infections (Kiesecker and Blaustein 1995; Kiesecker et al. 2001), while new metamorphs of these species, and also larvae in the case of *P. regilla*, seem to tolerate exposure to *Saprolegnia* zoospores (Romansic et al. 2006, 2007). Consequently, there is a need to investigate how *Saprolegnia* spp. affect amphibians at various life stages, which is important when evaluating the effects of these pathogens at the population level (Romansic et al. 2009).

Amphibian eggs are protected by a series of jelly layers and a fertilization coat that progressively degrades as the embryo grows and develops (Yamasaki et al. 1990). These coats have been suggested to act as a barrier against certain pathogens (Gomez-Mestre et al. 2006). This could mean that embryos are more susceptible to infections as they grow because of the lower protection provided by the jelly coats. To our knowledge, however, there are no studies regarding the development-dependent susceptibility of amphibian embryos to the pathogenic *Saprolegnia* spp.

In this work, the influence of the developmental stage throughout the embryonic period on the susceptibility to *Saprolegnia* infection was studied in two anuran species, the natterjack toad (*Bufo calamita*) and the Western spadefoot toad (*Pelobates cultripes*). These species usually breed in temporary ponds where they lay long strings containing several thousands of eggs. In addition, the pathogen *S. diclina* has already been isolated from dead *B. calamita* embryos in the field, and was shown to cause high embryonic mortality in this species (Fernández-Benéitez et al. 2008). In the case of *P. cutripes*, embryos affected by *Saprolegnia*-like infections are often found in the field (pers. obs.); however, no identification of *Saprolegnia* species that infect embryos of *P. cutripes* has been made to date.

Materials and methods

Saprolegnia isolation

Eggs of *B. calamita* and *P. cultripes* with signs of *Saprolegnia* infection were collected from two localities in the Sierra de Gredos (Ávila, Spain). The infected eggs are easily recognizable because they have a "cotton-like" appearance due to the growth of the *Saprolegnia* mycelium (Fernández-Benéitez et al. 2008). Eggs of *B. calamita* were collected at Prado de las Pozas (40°16′10″N, 5°14′47″W, 1,927 m above sea level), and eggs of *P. cultripes* were collected at Puerto del Tremedal (40°21′47″N, 5°36′35″W, 1,614 m above sea level). Isolations were carried out using colonized pieces of infected eggs, which were washed with distilled water containing 100 mg l^{-1} of penicillin C to prevent bacterial growth. A piece of infected egg was placed on top of peptone glucose agar (PG1), supplemented with 100 mg l^{-1} of penicillin C, in the inner area of a glass ring 3 cm in diameter. To minimize the risk of bacterial contamination when the mycelium crossed to the other side of the ring, it was transferred to a new PG1 plate. Isolates were maintained on PG1. Two isolates from *P. cultripes* and one from *B. calamita* were obtained and stored under the strain names of SAP436, SAP442 and SAP440, respectively, in the culture collection of the Real Jardín Botánico (Madrid, Spain).

The isolates were identified by analyzing rDNA-ITS sequences. For this purpose, mycelium was grown as drop cultures (Cerenius and Söderhäll 1985), and genomic DNA was extracted from these cultures using an E.Z.N.A. Fungal DNA Miniprep Kit (Omega Bio-Tek, Doraville, GA, USA). DNA fragments containing the internal transcribed spacers ITS1 and ITS2 including 5.8S were amplified with the primer pair ITS5/ITS4 (White et al. 1990), as described in Martín et al. (2004). Nucleotide BLASTN searches performed with the standard nucleotide BLAST (BLASTN 2.6) option were used to compare the sequences obtained with those available in the National Center for Biotechnology Information (NCBI) nucleotide databases.

Egg collection and virulence assay

Freshly oviposited eggs of *B. calamita* and *P. cultripes* [<24 h, stage <10 according to Gosner (1960)] with no signs of *Saprolegnia* infection were collected from four

different clutches of each species. The eggs were collected from the same locations as those from which the *Saprolegnia* strains were obtained. The eggs were transported to the laboratory and placed in 100 ml containers with 90 ml of commercial bottled mineral water. All experimental containers were kept in an aquarium with 30 l water maintained at 14°C with a Selecta 285 W refrigerator (J.P. Selecta SA, Abrera, Spain). The water temperature in the experimental containers was checked daily and found to vary by less than 1°C with respect to the temperature in the big aquarium. The photoperiod was established as 14:10 h light/dark cycles.

Eggs of each amphibian species were tested for susceptibility to zoospores of the specific *Saprolegnia* strains isolated from them. Thus, *B. calamita* embryos were exposed to the zoospores of the strain SAP440, and *P. cultripes* to the strains SAP436 and SAP442. Each 90 ml container had 20 eggs (5 eggs × 4 clutches) and was assigned to a developmental stage for the beginning of zoospore exposure (i.e., the developmental stage of the eggs at the time of zoospore addition) and, in *P. cultripes*, to a *Saprolegnia* strain. For developmental stage assignation, containers were divided into three groups, each corresponding to a developmental stage at the moment of zoospore addition (according to Gosner 1960): stage 12, corresponding to the late gastrula; stage 15, when the embryo rotates; and stage 19, when heart starts beating. Each treatment was replicated three times. Additionally, nine containers in each experiment were labeled as controls (no zoospores added) and randomly separated into three subgroups. Each subgroup was assigned to one of the three developmental stages, and was used as a control for that specific stage. The experiment began when all embryos were at stage 12, when zoospores were added to the first group. After 72 h, when all embryos were at stage 15, we added zoospores to the second group. After 144 h, when all the embryos were at stage 19, we added zoospores to the containers of the third group.

Zoospore production was performed as described by Diéguez-Uribeondo et al. (1994). Briefly, small pieces of mycelium were allowed to grow in drop cultures of PG1 for 72 h at room temperature (20°C). To remove the medium and induce sporulation, each culture was washed with autoclaved mineral water three times, with an hour interval between each wash. The zoospore harvest was performed 12 h after the last wash, and concentrations were calculated using a Blaubrand® Neubauer counting chamber (Brand, Wertheim, Germany).

The final zoospore concentrations in the *P. cultripes* experimental containers were 3,000 zoospores per ml for SAP436 and 15,000 zoospores per ml for SAP442. For *B. calamita*, we used two SAP440 zoospore concentrations: 300 and 3,000 zoospores per ml. These concentrations were selected on the basis of previous assays to determine the ability of the *Saprolegnia* strains to sporulate at room temperature (20°C). Briefly, for each *Saprolegnia* strain, three pieces of mycelium were grown in drop cultures of PG1 in each of 21 petri dishes. Sporulation was performed in the same way as for the infection experiments (see above), and the number of zoospores produced by each petri dish was recorded. The experimental concentration in each case was determined as the average zoospore concentration obtained for the pool of petri dishes. In the case of *B. calamita*, this procedure was followed to determine the higher zoospore concentration, and the lower one was established as one order of magnitude below the higher concentration.

The zoospores that we used for inoculation were obtained from a different set of petri dishes. In order to assure that there were sufficient zoospores to get the experimental concentrations, a minimum of 42 petri dishes per experiment were prepared. From the products of these petri dishes, we obtained a stock solution with a known zoospore concentration in order to add *Saprolegnia* to the containers. The concentrations of the stock solutions were 10,000 zoospores per ml in the *P. cultripes*-SAP436 experiment, 80,000 zoospores per ml in the *P. cultripes*-SAP442 experiment, and 16,000 zoospores per ml in the *B. calamita* experiment. Before the addition of zoospores, we carefully took out the same water volume as that of the stock solution to be added. This procedure was also used for controls, but mineral water instead of zoospore stock solution was added to the containers.

Containers were checked every 24 h, and the number of dead embryos after zoospore addition was recorded during 72 h. Dead embryos were checked for the presence of *Saprolegnia*. If *Saprolegnia* isolates were obtained, these were characterized molecularly to identify the species.

Data analysis

Mortality rates were calculated after 24, 48 and 72 h of zoospore addition. Mortality rates were arcsin of square root transformed before statistical analyses. To analyze the effect of zoospore concentration on embryonic survival in relation to developmental stage, we used a repeated measures analysis of variance (ANOVA) with the increase in mortality over time as the dependent variable and the developmental stage of the embryos at zoospore inoculation as the categorical factor. To assess the time after zoospore addition at which differences in mortality rates between the developmental groups appeared, we used one-way ANOVAs with the mortality rates every 24 h as dependent variables. The mortality rates of the three control subgroups were compared before this ANOVA, and no differences were observed ($F_{2,6} = 2.600$; $P = 0.154$).

Therefore, we pulled all the control containers together and used them as a single treatment within the ANOVA factor. Differences between specific treatments were checked with HSD Tukey post hoc tests. SPSS 11.5 for Windows® (SPSS Inc., Chicago, IL, USA) was used for statistical analyses.

Results

Saprolegnia characterization

Two different strains of *Saprolegnia* were identified from affected eggs of *P. cultripes* and stored in the culture collection of the Real Jardín Botánico (Madrid, Spain) as SAP436 and SAP442. The BLAST searches of the sequences from the *P. cultripes* egg isolates showed 100% similarity of SAP436 to GenBank sequence AM228844, corresponding to *S. diclina*, and 100% similarity of the isolate SAP442 to GenBank sequence AM228845, corresponding to *S. ferax*. With regards to *B. calamita*, the BLAST search of the sequence of the strain SAP440 showed 100% similarity to the sequence AM228844, corresponding to *S. diclina*. The new sequences were deposited in the GenBank collection.

Pelobates cultripes virulence assay

In the infection experiments with *P. cultripes* eggs, the average mortality in controls was less than 2%, symptoms of *Saprolegnia* infection were not observed, and *Saprolegnia* isolates were not obtained (Table 1). Overall mortality rates 72 h after zoospore addition were 64.9% in the embryos exposed to SAP436 (*S. diclina*) and 66.1% in those exposed to SAP442 (*S. ferax*).

Differences in zoospore sensitivity in relation to developmental stage were observed for both *Saprolegnia* species (Table 1). In all cases, embryos exposed at Gosner stage 12 showed a higher resistance to infection than those exposed at later stages. When embryos were challenged with *S. diclina*, the mortality rate of those exposed at stage 12 did not differ from that of controls after 72 h, and was only 3.3% at this time. Significant developmental stage-related effects appeared at 48 h after challenge ($F_{3,14} = 14.204$; $P < 0.001$). Post hoc tests revealed that, at that time, the mortality rates of embryos exposed at stage 15 (33.6%) and at stage 19 (66.7%) were significantly higher than that of controls (Fig. 1a). In spite of the big difference in mortality between the two sensitive developmental stages, the post hoc test failed to detect statistical differences between them.

With regards to *S. ferax*, we observed the same pattern as in *S. diclina*, with no effects on embryos exposed at stage 12 after 72 h, when the mortality was only 1.7%. In this case, significant lethal effects were also observed for the other two treatments at 48 h after challenge ($F_{3,14} = 282.622$; $P < 0.001$) (Table 1). At that time, 90.0% of the embryos exposed at stage 15 and 91.7% of those exposed at stage 19 had died (Fig. 1b).

In both experiments, isolates of the tested *Saprolegnia* species were obtained from dead embryos exposed to zoospores.

Bufo calamita virulence assay

In the *B. calamita* experiment, the average mortality in controls was less than 2%, and no symptoms of infection were observed. *Saprolegnia* could not be isolated from dead controls. Repeated measures ANOVA showed that the addition of SAP440 (*S. diclina*) zoospores increased the mortality of embryos, and this increase was dependent on

Table 1 Results of the repeated measures analyses of the variance performed to investigate the increases in the mortality rates of *Pelobates cultripes* and *Bufo calamita* embryos exposed at different developmental stages to strains of *Saprolegnia*

	Saprolegnia strain	Source of variation	*df*	Mean squares	*F*	*P*
P. cultripes	*S. diclina*	Zoospores	1	3.329	77.857	<0.001
		Stage	2	0.688	16.092	<0.001
		Zoospores × Stage	2	0.700	16.381	<0.001
		Error	12	0.043		
	S. ferax	Zoospores	1	4.554	548.819	<0.001
		Stage	2	1.275	153.678	<0.001
		Zoospores × Stage	2	1.167	140.622	<0.001
		Error	12	0.008		
B. calamita	*S. diclina*	Zoospores	2	3.074	13.448	<0.001
		Stage	2	2.831	12.383	<0.001
		Zoospores × Stage	4	0.738	3.229	0.037
		Error	18	0.229		

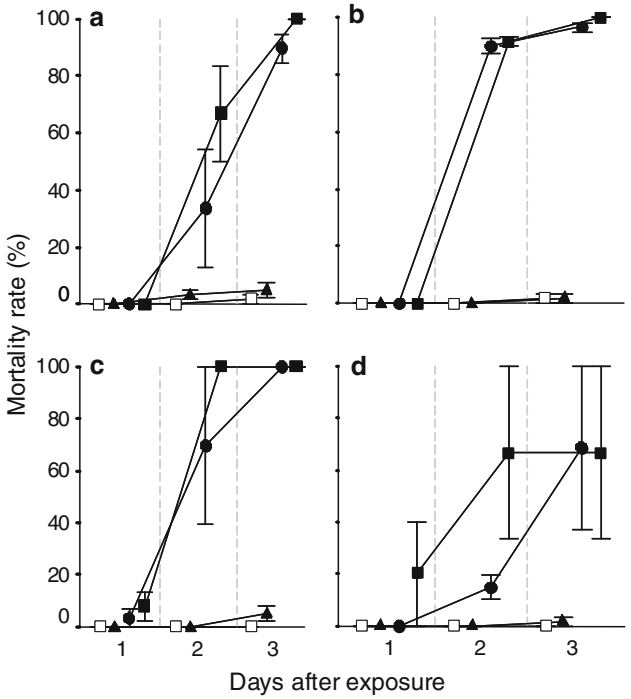

Fig. 1a–d Mortality rates (mean ± SE) of embryos exposed to zoospores of *Saprolegnia* at different developmental stages according to Gosner (1960). **a** *Pelobates cultripes* embryos exposed to 3,000 zoospores per ml of *Saprolegnia diclina*, **b** *Pelobates cultripes* embryos exposed to 15,000 zoospores per ml of *Saprolegnia ferax*, **c** *Bufo calamita* embryos exposed to 3,000 zoospores per ml of *Saprolegnia diclina*, and **d** *Bufo calamita* embryos exposed to 300 zoospores per ml of *Saprolegnia diclina*. *Open squares* represent control embryos (no zoospores added), *black triangles* represent embryos exposed at stage 12, *black circles* represent embryos exposed at stage 15, and *black squares* represent embryos exposed at stage 19. In those treatments with 0 or 100% mortality, no error bars can be calculated given that mortality was consistent for all replicates. The datapoints for each treatment are offset for visibility

the embryonic developmental stage at which zoospores were added (Table 1).

As in the previous experiment, a decreased effect of the pathogen was observed in embryos exposed at developmental stage 12, with a mortality rate of 2.2% at 72 h after zoospore inoculation. Differences in mortality rates among developmental stage groups appeared after 48 h of exposure at the highest zoospore concentration ($F_{3,14} = 28.806$; $P < 0.001$). At that time, all of the embryos exposed at stage 19 and 69.7% of those exposed at stage 15 had died (Fig. 1c). For the lowest zoospore concentration, differences in mortality rates appeared 48 h after zoospore addition ($F_{3,14} = 6.497$; $P = 0.006$), although in this case only embryos exposed at stage 19 suffered from a mortality rate that was significantly higher than the controls. After 72 h of exposure, development-related differences in mortality rate were also recorded for embryos exposed at stage 15 ($F_{3,14} = 6.641$; $P = 0.005$), when 68.5% of them had died (Fig. 1d). Although there is a trend indicating that embryos exposed at stage 19 were more sensitive than those exposed at stage 15, the post hoc test did not reveal any significant difference between these two groups at any concentration or exposure time.

Isolates of *Saprolegnia diclina* were obtained from dead embryos exposed to the pathogen.

Discussion

The present study shows that both *B. calamita* and *P. cultripes* embryos are susceptible to lethal infections by the *Saprolegnia* species isolated from dead eggs in their natural habitats. The results from the *B. calamita* experiment confirm those of Fernández-Benéitez et al. (2008), which demonstrate that *S. diclina* is a primary pathogen in embryos of this species. In addition, this is the first study that identifies both *S. diclina* and *S. ferax* as causative agents of *P. cultripes* embryonic die-offs due to *Saprolegnia* infections. While the virulence of *S. diclina* towards amphibians has only been demonstrated—to the best of our knowledge—in the two species analyzed in the present paper, *S. ferax* has been cited as a pathogenic agent for a wider number of amphibian species (e.g., Kiesecker and Blaustein 1999).

We report a dose–response relationship for the effects of different zoospore concentrations in the *B. calamita* experiment in terms of both final mortality and time for symptoms to occur. Most studies regarding the effects of *Saprolegnia* spp. on amphibians use pieces of mycelium as infecting vectors without further quantifying the amount of zoospores that can be involved in the infection process (e.g., Gomez-Mestre et al. 2008; Sagvik et al. 2008b; Karraker and Ruthig 2009). In these cases, although a consistency in sporulation among mycelium pieces can be assumed, the identification of lethal or sublethal pathogen densities may become difficult. To the best of our knowledge, only Romansic et al. (2007) and Fernández-Benéitez et al. (2008) quantified the initial number of *Saprolegnia* zoospores and zoospore cysts. Whereas the former study used a single experimental concentration, the latter one reported, as in the present paper, a dose–response relationship between the zoospore concentration of *S. diclina* and the mortality rate of *B. calamita* embryos. These results highlight the importance of knowing zoospore concentrations in order to establish pathogen virulence with accuracy.

Embryonic developmental stage seems to play a primary role in the sensitivity of both amphibian species to *Saprolegnia* infection. Embryos challenged at Gosner stages 15 and 19 suffered increased mortality after 72 h of exposure, while those exposed at Gosner stage 12 were

tolerant of the effects of *Saprolegnia* at this time. The current study is the first that experimentally analyzes the sensitivity to *Saprolegnia* spp. in particular, and oomycetes in general, across embryonic development in amphibians.

Blaustein et al. (1994) observed in the field that embryos of *B. boreas* developed normally until stage 13. At this stage, hyphae became clearly visible on the embryos and began to grow outward through the vitelline membrane. These authors, however, also observed that mortality was especially high when embryos were infected by *Saprolegnia* before the development of the neural crest (Gosner stage 16). Thus, they proposed that individuals infected after this stage could stand the pathogen challenge. According to this hypothesis, in our study, embryos exposed at stage 19 should have tolerated the effects of *Saprolegnia*. However, in all cases, we observed significant mortality of embryos exposed at this developmental stage 72 h after zoospore addition. Therefore, our results demonstrate that neural crest formation is not a critical stage for *Saprolegnia* infections, at least under the conditions used in our experiments.

One of the main factors determining the variation in the tolerance to pathogens throughout the ontogeny is the stage of development of the immune system. There are few studies on amphibian developmental immunology. Du Pasquier et al. (1989) found that the immunological response in *Xenopus laevis* starts to function after hatching. On the other hand, Poorten and Kuhn (2009) demonstrated the existence of maternal transfer of antibodies in the same species. If the organisms are unable to show an immune response until hatching and/or they rely on maternal antibodies, no changes in the sensitivity to pathogens related to these immune skills are expected to occur during embryonic development. Furthermore, if some immunological responses were developed during the embryonic phase, later stages would be more tolerant of pathogens than earlier ones, which is contrary to what we observed. Therefore, other mechanisms may play a role in the resistance of young embryos to *Saprolegnia*.

The reported developmental differences in sensitivity to *Saprolegnia* infections could be a consequence of the changes in the thickness of the jelly cap that surrounds the embryo. This gelatinous matrix may act as a barrier that protects eggs from the pathogen during the early stages, when the jelly coat is especially thick. As the embryos develop, this cover becomes thinner and thus physical contact with the pathogen is facilitated. Gomez-Mestre et al. (2006) found that *Ambystoma maculatum* eggs with intact jelly caps were resistant to infection by water molds belonging to the genera *Saprolegnia* and *Achlya*, while eggs whose jelly coats were removed suffered high mortality rates. Therefore, the higher sensitivities of later embryonic stages to *Saprolegnia* may be attributed, at least in part, to the higher protective effect of the jelly coat during the early stages, when this coat is thicker.

Other potential defences against fungal infections, such as the substances derived from symbiotic bacteria described for the embryos of some taxa (see review in Hamdoun and Epel 2007), have not been studied in amphibian embryos. However, it has been observed that in some molluscs those symbiotic bacteria that protect against fungi are usually associated with the jelly coat that covers the egg (Kaufman et al. 1998). Further research is needed to establish if the jelly coat in amphibians might be hosting some symbiotic microbes that could be protecting embryos against infection, and also to check how this protection may vary throughout development.

The reported differences in susceptibility to *Saprolegnia* at various developmental stages may be important for understanding the effects caused by *Saprolegnia* when combined with other stressors, such as ultraviolet B radiation (UV-B) or pollutants. For example, environmental increases of inorganic nitrogen have been suggested to be related to the outbreaks of several amphibian diseases (Johnson et al. 2007). However, the few studies analyzing the combined effects of *Saprolegnia* and inorganic nitrogen performed so far have not found clear evidence of synergistic effects (Romansic et al. 2006; Puglis and Boone 2007). Nevertheless, Ortiz-Santaliestra et al. (2006) demonstrated that, when exposing embryos and early larvae of several amphibian species—including *P. cultripes* and *B. calamita*—to ammonium nitrate, age variations of only four days caused big differences in the sensitivity of individuals. Furthermore, in the case of *P. cultripes*, animals exposed at Gosner stage 19 were the most sensitive, as observed in the *Saprolegnia* challenges described in this paper. The occurrence of combined effects of *Saprolegnia* and inorganic nitrogen could therefore be dependent on the developmental stage at which the animals are exposed.

In contrast to what happens with inorganic nitrogen, the synergistic effects of UV-B and *Saprolegnia* on amphibian embryos have been demonstrated (Kiesecker and Blaustein 1995). In some species, the embryo jelly coat appears to absorb wavelengths in the UV-B range (Ovaska et al.1997), thus playing an important role in determining the amount of damaging UV-B radiation that reaches the embryo (Smith et al. 2002). As the embryo grows and the jelly coat becomes thinner, its efficiency at blocking ultraviolet light is expected to diminish, and thus the later embryonic stages might be more sensitive not only to the impact of *Saprolegnia* but also to the deleterious effects of UV-B. If sensitive stages to two stressors that act synergistically are coincident, the effects of these stressors will be strongly magnified if they appear in the field at the same time.

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