

EVALUATION OF STRESS RESPONSES TO WATER MOLDS IN EMBRYOS OF *PHYSALAEMUS ALBONOTATUS* (ANURA: LEPTODACTYLIDAE)

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Abstract.—Infections by water molds of the genus *Saprolegnia* (Oomycetes) have been associated with the death of eggs and embryos in many anuran species; however, how water molds induce physiological responses in early and susceptible developmental stages of anurans is poorly known. To assess whether or not embryos of *Physalaemus albonotatus* (immobile and susceptible life stages) respond to Oomycetes infection, we performed a 4-d experiment exposing early stage embryos to the water mold *Saprolegnia*-like sp. We assessed mortality, hatching time, and oxidative stress (antioxidant enzyme activities and lipid peroxidation levels). Presence of water molds in infected embryos was not evident to the naked eye until 1 to 1.5 d after inoculation. Mortality was significantly higher in the water mold treatment than in the control treatment. Embryos exposed to *Saprolegnia*-like sp. hatched 24 h earlier than those in control treatment. Among enzymatic activities, only catalase enzyme showed a significant depletion in embryos exposed to water molds compared to control groups. These results emphasize the need to explore oxidative stress markers along with embryonic development at key stages, focusing on those early stages where hatching is induced by water molds infection.

Key Words.—amphibian embryo; hatching time; mortality; oxidative stress; *Saprolegnia* sp. infection

INTRODUCTION

The maintenance of homeostasis in an individual may be affected by natural stressors (Burraco and Gomez-Mestre 2016; Freitas and Almeida 2016). Homeostatic adjustments may trigger cascading effects along multiple physiological pathways, affecting, for example, the metabolic rate increasing metabolic demands in multiple tissues (Pecket et al. 2011), the immune system resulting in an increased neutrophils:lymphocytes ratio (Davis et al. 2008), and/or the reactive oxygen species (ROS) production involving over production of ROS (Circu and Aw 2010). The overproduction of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, and enzyme inhibition, leading ultimately to cell death (Burraco and Gomez-Mestre 2016). Such deleterious effects of ROS can be neutralized by antioxidant defense systems, keeping ROS at relatively low levels and repairing or removing the damaged molecules (Pinya et al. 2016). The antioxidant defense system includes endogenous antioxidants (enzymatic and non-enzymatic) such as superoxide dismutase (SOD), catalase (CAT),

glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferases (GST) among others, and exogenous antioxidants such as vitamin C, vitamin E, and carotenoids, derived primarily by eating animals (Halliwell and Gutteridge 2007).

Primary enzymatic antioxidants such as GR, CAT, and general peroxidases are especially adept at diminishing or neutralizing ROS (Halliwell and Gutteridge 2007). Glutathione reductase is primarily responsible for maintaining high glutathione (GSH) levels in the GSH/GSSG (glutathione disulfide) ratio, favoring GSH for the reduction of oxidized ascorbate and other antioxidant roles (Foyer et al. 1991). Catalase catalyzes excess H₂O₂ to H₂O and O₂ (Chang et al. 1984). A variety of general peroxidases degrades hydrogen peroxide (Jones et al. 2010).

In these compensatory mechanisms, under several stressful conditions, the rate of ROS production can exceed the capacity of antioxidant defense, leading to a condition known as oxidative stress (Halliwell and Gutteridge 2007). The application of oxidative stress biomarkers in assessing stress in animals exposed to different environmental situations may show what strategies these organisms have developed to cope with

different stress conditions (Almeida and Mascio 2011; Freitas and Almeida 2016). In fact, several studies have demonstrated that amphibians exposed to environmental stressors (e.g., pesticides, predators, low pH) can trigger an excess of ROS, inducing antioxidant activation (Attademo et al. 2007; Jones et al. 2010; Burraco and Gomez-Mestre 2016).

Water molds infecting amphibians and functioning as naturally occurring stressors include many facultative pathogens such as species of the genera *Achlya*, *Leptolegnia*, *Aphanomyces*, and *Saprolegnia* (Gleason et al. 2014), all of which have been recognized as important pathogens of amphibians since the mid-20th Century (e.g., from Bragg 1958 to Bovo et al. 2016). Infection by *Saprolegnia* spp. is associated with reduced hatching time and higher mortality rates of many amphibian embryos (Bragg 1958, 1962; Blaustein et al. 1994; Fernández-Benítez et al. 2011; Perotti et al. 2013). Decreased hatching time shortens the developmental period of embryos and reduces mass at metamorphosis, both of which have important implications for anuran population dynamics (Touchon et al. 2006; Uller et al. 2009; Perotti et al. 2013).

To date, several studies have demonstrated the effects of infection by water molds (Oomycetes) on survival and development in amphibians (Blaustein et al. 1994; Fernández-Benítez et al. 2008, 2011; Ruthig 2009; Perotti et al. 2013), linking fungal-like infections with mortality or shortening of hatching time (Gomez-Mestre et al. 2006; Touchon et al. 2006; Perotti et al. 2013). However, no studies have determined whether or not amphibian embryos under the risk of Oomycetes infection trigger antioxidant activation. This information would complement other studies of Oomycetes effects on anuran larvae because multiple assessments of the physiological state of animals are important to understand the basis and consequences of phenotypic plasticity (Burraco et al. 2013).

Physalaemus albonotatus (Anura: Leptodactylidae) is a South American species that breeds during spring and summer in temporary wetlands and flooded grasslands (López et al. 2011). Females typically lay 600–1,500 eggs immersed in foam nests (Ghirardi and López 2016). Temporary ponds that support *P. albonotatus* reproduction are generally also suitable habitat for Oomycetes, and *Saprolegnia* sp. has been found on live and decaying plant and animal material, from which it is able to infect insects, reptiles, fish, and the eggs of fish and amphibians (Gomez-Mestre et al. 2006).

The presence of Oomycetes at our study sites motivated us to study how the immobile and more susceptible stages (i.e., eggs and embryos) of *P. albonotatus* respond to the stress imposed by infection with water molds (*Saprolegnia* sp.). We predicted

that the embryonic mortality rates would increase, and embryos that survived to hatching would exhibit reduced hatching time (ostensibly as a mechanism to evade the risks of heavy infection loads). We also predicted that there would be allostatic adjustments to shift the production of ROS. Therefore, we expected an increase in activity of antioxidant enzymes as a means of increasing defense systems of embryos.

MATERIALS AND METHODS

Amphibian egg and water mold collection.—In November 2014, we collected *Physalaemus albonotatus* clutches and samples of water molds in a semi-permanent pond from the alluvial valley in the Middle Paraná River, near Santa Fe, Argentina (31°37'S, 60°41'W). The pond was approximately 90 m diameter, 0.20 m deep, and covered by Alligator Weed (*Alternanthera philoxeroides*). We collected the clutches by hand and kept them in plastic dishes filled with 1 L chlorine-free water. We also brought 0.5 L of pond water to the laboratory for Oomycetes identification.

Oomycetes isolation and egg maintenance.—To isolate Oomycetes, we distributed water samples in sterile Petri dishes (10 cm diameter with 40 mL of pond water each) with sterile Sesame seeds (*Sesamum indicum*) added as a bait substrate. We incubated them in an incubator under controlled temperature and photoperiod conditions (20 ± 1° C; 12:12 h L:D). We visually monitored and scored mycelium colonization 48–72 h after inoculation. After water mold grew on the seeds, we transferred a clump to corn meal agar medium (Emerson YPSS Agar) and augmented it with two antibiotics (Chloramphenicol and Streptomycin-sulphate) to avoid bacterial growth (Promega Corporation 2007). To ensure axenic cultures, we transferred mycelium tips to new media twice. After 3–4 d, we cut off a block of agar (1 × 1 cm) from the edge of each colony and placed it in a sterilized Petri dish containing sterilized water and seeds to let the mycelium grow (Robinson et al. 2003). After 4–5 days of incubation, the seeds were sufficiently colonized (about 1 cm diameter) to perform the experiment. We identified water mold to genus as *Saprolegnia* sp. (hereafter referred to as a *Saprolegnia*-like sp; Fig. 1A) under a stereoscopic microscope and following Seymour (1970) and Fuller and Jaworski (1987). We used these colonized seeds with *Saprolegnia*-like sp. clumps to inoculate the infection-treatment in the water mold exposure experiment (Romansic et al. 2009). We collected fresh eggs of *P. albonotatus* that were approximately 12 h old, and at stages 13–14 (Gosner 1960) from four clutches with no signs of water mold infection. To remove potential presence of Oomycetes,

we carefully extracted eggs from the foam matrix and washed them for approximately 10 s in boiled pond water before setting up the experiment (Ruthig 2009).

Experimental setup.—We performed a single experiment to test if *Saprolegnia*-like sp. infection affects mortality, hatching time, and activation of the antioxidant system of *P. albonotatus* embryos. The experiment was a single-factor design with *Saprolegnia*-like sp. infection as a fixed factor. Each experimental unit consisted of a Petri dish (10 cm diameter, 1 cm high, 40 mL of tap water) plus two sterilized Sesame seeds in the control group and two Sesame seeds infected with *Saprolegnia*-like sp. in the infection treatment group. To each Petri dish we added 10 embryos randomly selected from among four clutches to avoid confounding effects of genetics. We arranged the eggs individually in the Petri dishes, evenly spaced, and without contact among them. We used 20 replicates per treatment, for a total of 40 experimental units, and ran the experiment in an incubator with controlled temperature and photoperiod ($21 \pm 1^\circ\text{C}$; 12:12 h L:D; Ruthig 2009).

The experiment concluded when almost 95% of the embryos hatched or died, which occurred over an approximately 4 d period, which is the embryonic development time in *P. albonotatus* (Romero-Carvajal et al. 2009; Gómez et al. 2016). We visually examined the Petri dishes daily and counted healthy, dead, and hatched embryos. The infected embryos were easily recognizable by their cotton-like appearance (Fig. 1B) caused by proliferation of *Saprolegnia*-like sp. mycelium (Fernández-Benítez et al. 2008). At each observation, we removed all dead embryos and, at the end of the experiment, we immediately froze surviving embryos in liquid nitrogen and stored them at -80°C for later determination of oxidative stress markers.

Determination of oxidative stress markers.—We determined oxidative stress markers from pools of whole-body individuals. We excluded deceased animals from the Petri dishes at each 24-h observation interval. Then, we combined surviving animals and used them for stress evaluation (5–10 individuals per Petri dish).

For enzyme extraction, we homogenized each group of embryos in an ice-cold 0.1-M sodium phosphate buffer, pH 6.5, containing 20% (v/v) glycerol, 1 mM EDTA, and 1.4 mM dithioerythritol. We centrifuged the homogenates at $20,000 \times g$ (4°C) for 30 min, and the supernatant was stored at -80°C for enzymatic measurements. We calculated the activity of glutathion S-transferase according to the method described by Habig et al. (1974), which assesses the formation of the conjugate of two substrates (1 mM reduced glutathione and 1 mM 1-chloro-2,4-dinitrobenzene) catalyzed by GST in the homogenate. We measured the reaction for

3 min at 340 nm and expressed the enzyme activity as $\text{nkat}\cdot\text{mg}\cdot\text{prot}^{-1}$ (prot = protein) using a molar extinction coefficient of $9.6\text{ mM}^{-1}\text{ cm}^{-1}$.

We estimated the glutathione reductase activity by using the method described by Tanaka et al. (1994). This spectrophotometric assay involves the reduction of glutathione disulfide (20 mM GSSG) to reduce glutathione by monitoring oxidation of NADPH (2 mM NADPH). We measured the activity for 3 min at 340 nm and it was expressed as $\text{nkat}\cdot\text{mg}\cdot\text{prot}^{-1}$ using a molar extinction coefficient of $6.22\text{ mM}^{-1}\text{ cm}^{-1}$. We calculated catalase activity according to Beutler (1982). We assayed the rate of decomposition of hydrogen peroxide by the enzyme (in the homogenate) using Tris buffer (1 M, pH 8.0) with 5 mM EDTA and 10 mM H_2O_2 as the substrate. We measured the enzyme activity at 240 nm for 5 min and it was expressed as $\text{nkat}\cdot\text{mg}\cdot\text{prot}^{-1}$ using a molar extinction coefficient of $0.071\text{ mM}^{-1}\text{ cm}^{-1}$. We assayed each enzyme measurement in triplicate and reported the activity as $\text{nkat}\cdot\text{mg}\cdot\text{prot}^{-1}$. We measured the protein content of the homogenate, using bovine serum albumin as standard protein (Sigma, St. Louis, Missouri, USA; Bradford 1976).

To evaluate cellular damage, we used the thiobarbituric acid (TBARS) method described by Fatima et al. (2000) to estimate the peroxidation of lipids (LPO). Briefly, we homogenized pools of whole-body hatchlings with 0.15-M potassium chloride solution using a glass homogenizer. Then, we incubated 1.0 mL homogenate for 1 h at 37°C with continuous shaking. Afterwards, we added 1.0 mL of 5% trichloroacetic acid, and 1.0 mL of 0.67% thiobarbituric acid to each sample and mixed them. Then, we centrifuged each vial at 3,000 rpm for 10 min. We separated the supernatant and placed it in a boiling water bath for 10 min, cooled to room temperature, and measured absorbance at 535 nm. We expressed the rate of lipid peroxidation as nanomoles of substances reactive to thiobarbituric acid (TBARS) formed per hour, per milligram of proteins ($\text{nmol TBARS}\cdot\text{mg}\cdot\text{prot}^{-1}$). We determined protein content of each homogenate according to Bradford (1976).

Statistical analysis.—To analyze the effect of water mold infection in embryos of the anuran *Physalaemus albonotatus*, we measured mortality (daily number of dead embryos) and hatching time (number of days from the beginning of our experiment until the emergence of each hatchling from its egg) as response variables. We used treatment (control/mold) and day as fixed predictor variables and treatment replicate (Petri dish) as a random predictor variable. Because the data fit a Gaussian distribution, we applied Mixed Linear Models (MLM). When effects of predictor variables were significant, we performed *post-hoc* multiple comparisons applying

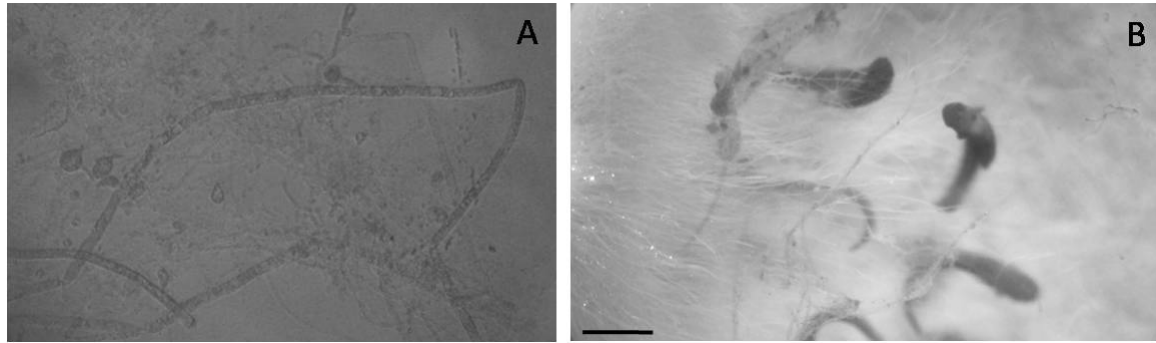


FIGURE 1. (A) Microscopic slide glass preparation with dense mycelium of *Saprolegnia*-like sp. Objective lens: 20 \times . (B) Infected *Physalaemus albonotatus* embryos with zoospores of *Saprolegnia*-like sp. The pathogen grows as cotton-like masses. Scale bar = 2 mm. (Photographed by Romina Ghirardi).

Tukey Contrasts tests. When data did not meet the statistical assumptions required for the analysis (e.g., homoscedasticity and normality), we performed a log10 transformation to improve the fit of the residuals, which met parametric assumptions.

We tested enzyme activity and lipid peroxidation via Mann-Whitney *U* tests using GST, GR, CAT, and LPO as response variables and treatment (control/mold) as fixed predictor variables. We applied a sequential Bonferroni correction to control the family-wise Type 1 error rate ($\alpha = 0.0125$). We performed analyses with package multcomp (Hothorn et al. 2008) and nlme (Pinheiro et al. 2015) for R version 3.3.3 (R Core Team 2017).

RESULTS

Embryo mortality and hatching time.—Presence of water molds in infected embryos was not evident to the naked eye until 1–1.5 d after inoculation. All dead embryos exposed to water mold treatments exhibited *Saprolegnia*-like sp. infections (Fig. 1B). The mortality model showed significant interaction between fixed

predictor variables (treatments and days; $F_{3,114} = 8.97$, $P < 0.001$). Mortality differed significantly among days ($F_{3,114} = 17.54$, $P < 0.001$), with day 1 significantly lower than days 2, 3, and 4 (Tukey Contrasts test, $P < 0.001$). Mortality also differed significantly between treatments ($F_{1,38} = 12.6$, $P = 0.001$). At the end of the experiment, 13.5% of embryos exposed to water mold died, whereas just 2% of individuals died in the control group (Fig. 2).

The hatching model showed significant interactions between fixed predictor variables (treatments and days; $F_{3,114} = 53.78$, $P < 0.001$). Hatching time differed significantly among days ($F_{3,114} = 813.6$, $P < 0.001$), with the number of hatched embryos different in day 1 from those of days 2, 3, and 4; and day 2 from days 3 and 4 (Tukey Contrasts test, $P < 0.001$). Hatching time also differed significantly between treatments ($F_{1,38} = 51.53$, $P < 0.001$). In the water mold treatment, hatching began a day earlier than in the control treatment, and reached 30% by the second day of experiment, and nearly 90% on the third day (Fig. 3). Hatching of embryos in the control treatment was explosive, with more than 80% of embryos hatching simultaneously and later than mold

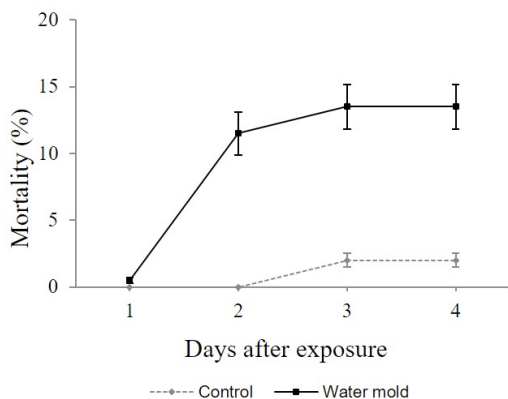


FIGURE 2. Mortality (mean \pm SE) of *Physalaemus albonotatus* embryos exposed to *Saprolegnia*-like sp. Sample size was 200 embryos per treatment.

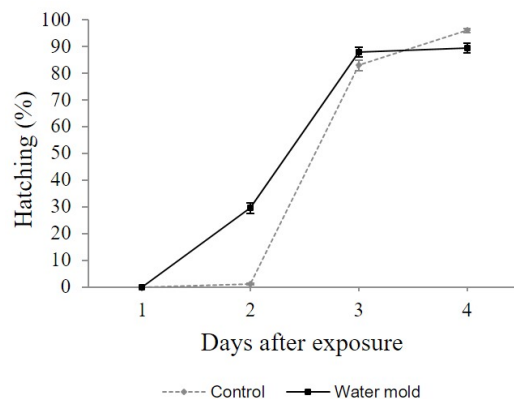


FIGURE 3. Hatching events (mean \pm SE) of *Physalaemus albonotatus* embryos exposed to water mold *Saprolegnia*-like sp. Sample size was 200 embryos per treatment.

TABLE 1. Activity of glutathione S-transferase (GST), glutathione reductase (GR), catalase (CAT), and levels of lipid peroxidation (LPO) in embryos of *Physalaemus albonotatus* from control and water mold treatments. GST, GR, and CAT are expressed as $\text{ngat}\cdot\text{mg}\cdot\text{prot}^{-1}$ and LPO as $\text{nmol TBARS}\cdot\text{mg}\cdot\text{prot}^{-1}$ (TBARS refers to thiobarbituric acid; prot refers to protein). The values are expressed as means \pm SE. Sample size was five per treatment.

	Control	Water mold
GST	22.18 \pm 1.69	21.75 \pm 0.91
GR	1.45 \pm 0.22	1.51 \pm 0.40
CAT	589.76 \pm 49.66	362.43 \pm 31.29
LPO	0.25 \pm 0.08	0.29 \pm 0.07

treatment (no embryos in control dishes hatched before the third day; Fig. 3).

Oxidative stress markers.—Neither glutathione S-transferase nor glutathione reductase were influenced by the exposure at *Saprolegnia*-like sp. (GST: $W = 14$, $P = 0.840$; GR: $W = 13$, $P = 1.00$; Table 1). Only catalase showed a significant depletion in embryos exposed to water molds compared to control groups (CAT: $W = 25$, $P = 0.007$). The TBARS used here as a proxy for general measure of oxidative damage, did not vary with presence or absence of water mold (LPO: $W = 6$, $P = 0.780$).

DISCUSSION

The increased attention on the impact of fungal and fungal-like pathogens in amphibian mortality events and amphibian population dynamics has contributed to the knowledge of their effects on different anuran species and their responses (Fernández-Benéites et al. 2011; Perotti et al. 2013; Burraco et al. 2017). In this work, we evaluated the responses of early developmental stages (Gosner stages 13–14) of *Physalaemus albonotatus* exposed to *Saprolegnia*-like sp. In our study, we found high mortality of embryos exposed to water mold compared to embryos not exposed; however, mortality rates were lower than in other studies (Villa 1979; Ruthig 2009; Perotti et al. 2013). Additionally, we observed that *P. albonotatus* embryos adjusted their hatching time, accelerating embryonic development (24 h ahead of their usual 4 d development) when they were at risk of infection by *Saprolegnia*-like sp.

During advanced embryonic stages (Gosner stages 18–19), the jelly coat becomes thinner and it has been suggested that embryos may develop alternative and effective defense strategies against water mold and true fungus infections such as accelerated hatching (Warkentin et al. 2001; Gomez-Mestre et al. 2006; Touchon et al. 2006; Perotti et al. 2013). This early hatching strategy is common in amphibians, allowing them to cope with the risk imposed by stressors such as water mold infections (Perotti et al. 2013), predation

risk (Chivers et al. 2001; Vonesh 2005), and desiccation (Gomez-Mestre et al. 2013; Salica et al. 2017).

To our knowledge, there are no studies focusing on potential homeostatic adjustments in response to the stressful conditions caused by water molds. The evaluation of ROS showed a significant depletion of catalase enzyme in surviving embryos in the water mold treatment compared to embryos from the control group. Catalase is a common enzyme, usually located in peroxisomes, and is an important ROS-scavenging enzyme during stress conditions (Pinya et al. 2016). Decreased catalase activity observed in our study could be the result of oxidative inactivation by free radicals (Pigeolet et al. 1990) due to reduced *de novo* synthesis (Kaushik and Kaur 2003).

Jones et al. (2010) reported higher catalase activity in liver than in muscle tissue in *Lithobates catesbeianus* tadpoles exposed to Paraquat contaminant, supporting the idea that the estimation of oxidative stress based only on muscle tissue could fail to reveal effects occurring in liver tissue. Similarly, Hermes-Lima et al. (1998) and Lushchak et al. (2001), studying snails and fish, respectively, suggested that liver tissue is more active than skeletal muscle in oxidative protection measures. Further studies considering only liver tissues will be necessary to estimate oxidative activity after water mold infection. It is likely that our estimation of enzyme activity based on the whole-organism analyses underestimates rates in relevant organs because of dilution with unrelated tissues. It is important to note that although we detected reduced antioxidant activity (CAT), it did not seem to come at the cost of increased oxidative damage, because TBARS used as a proxy for general measure of oxidative damage did not vary in the presence or absence of *Saprolegnia*-like sp. However, considering the widespread distribution of water molds and the potential risk they present to amphibians, we stress the need to explore oxidative stress markers on the different susceptible stages of amphibians, and examine them through key developmental stages.

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