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EGG-KILLING FUNGUS INDUCES EARLY HATCHING OF RED-EYED TREEFROG EGGS

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Abstract. Pathogens can cause substantial mortality of amphibian eggs. If the timing of hatching is phenotypically plastic, embryos could escape from otherwise lethal infections by hatching early. We tested this with the arboreal eggs of red-eyed treefrogs, *Agalychnis callidryas.* A filamentous ascomycete (Dothideales: Phaeosphaeriaceae) was present on ~7% of egg clutches collected from a pond in the rain forest in Panama and, when present, killed 40% of the eggs, on average. Inoculation experiments confirmed that the fungus attacked and killed healthy embryos, establishing that this fungus is a pathogen of *A. callidryas* eggs. As predicted from life history theory, embryos hatched earlier from both naturally infected and inoculated clutches than from fungus-free control clutches. Within infected clutches, live embryos in contact with fungal hyphae hatched before those embryos not in contact with the fungus. Accelerated hatching allowed embryos to survive that otherwise would have been killed, and tadpoles hatched from infected clutches were themselves uninfected. Red-eyed treefrog embryos also hatch early if attacked by predators, apparently in response to vibratory cues. Because fungal infection provides no vibratory stimuli, embryos must respond to different cues in fungus-induced hatching than in predator-induced hatching. The behavioral decision of when to hatch is complex and merits further investigation. Our study indicates that pathogens can influence the timing of life history transitions, as do other stage-specific risks.

Key words: Agalychnis callidryas; *anuran; Dothideales; fungal infection; ontogenetic niche shift; Panama; pathogen; Phaeosphaeriaceae; phenotypic plasticity; red-eyed treefrog; tadpole; timing of hatching.*

INTRODUCTION

Life history theory predicts that the timing of transitions between different life stages should vary with the costs and benefits accruing in the current and succeeding stages (Werner and Gilliam 1984, Werner 1986, 1988). Thus the timing of hatching, the transition between egg and larval stages, should vary with risk of mortality to embryos and larvae (Sih and Moore 1993, Warkentin 1995, 1999*a*). Abiotic stresses affect the timing of hatching as predicted (reviewed in Martin 1999), and variation in hatching stage in response to egg and larval predators has recently been discovered (Sih and Moore 1993, Warkentin 1995, 2000). Pathogens also cause substantial mortality of eggs and larvae (e.g., Banks and Beebee 1988, Blaustein et al. 1994, Williamson and Bull 1994); however, their effects on the timing of hatching have not been examined. Pathogens have important effects on host life history, behavior, and population dynamics (Dobson and Hudson 1986, Minchella and Scott 1991, Dobson and Crawley 1994, Poulin 1994). Their ecological significance is probably greater than the relative amount of attention that they have received from ecologists (Price et al. 1986, Marcogliese and Cone 1997).

Pathogens, including several species of fungi and water molds, can pose a substantial risk to amphibian embryos (Blaustein et al. 1994, Czeczuga et al. 1998). In many natural amphibian populations, fungal infections of eggs impose moderate levels of mortality (e.g., Forester 1979, Simon 1983, Kam et al. 1996). Fungi and water molds pathogenic to eggs can impose selection on oviposition site choice, egg distribution (degree of clumping), and parental care behavior (Forester 1979, Kiesecker and Blaustein 1997, Green 1999). These pathogens are also implicated as a cause of amphibian population declines, in combination with environmental stresses such as low temperature and pH and high UV-B radiation that magnify the lethal effects of pathogen infections (Banks and Beebee 1988, Bellemakers and van Dam 1992, Kiesecker and Blaustein 1995).

Recent studies of amphibian embryos indicate that egg predators can induce changes in hatching stage. The salamander *Ambystoma barbouri* delays hatching when exposed to larval predators (Sih and Moore 1993, Moore et al. 1996). Red-eyed treefrogs, *Agalychnis callidryas,* hatch early in response to egg-eating snakes and wasps (Warkentin 1995, 2000). The frogs *Hyla*

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regilla and *Rana cascadae* hatch early in response to egg-eating leeches (Chivers et al. 2001), and *Hyperolius cinnamomeoventris* hatches at a smaller size when exposed to egg-eating fly larvae (Vonesh 2000). Embryos also respond to abiotic factors affecting egg and larval survival. At least five species of fish and four of amphibians delay hatching under physical conditions suitable for egg development, but unsuitable for larval survival (reviewed in Martin 1999). If hatchlings can escape from the fungal infections that kill eggs, accelerated hatching of embryos in infected clutches would be advantageous. We test for an effect of a lethal fungus on hatching timing in an amphibian known to exhibit plasticity in hatching.

Red-eyed treefrogs inhabit lowland wet forests from the Yucatan through Panama. They typically breed at long-lasting seasonal ponds and swamps, attaching egg clutches to vegetation overhanging the water. After up to 10 d of embryonic development, tadpoles hatch and fall into the water. Hatching stage is variable, and survival of hatchlings with aquatic predators improves with hatching age (Warkentin 1995, 1999*b*). Most undisturbed eggs hatch late but young embryos physically disturbed by egg predators, or certain artificial disturbances, respond by immediate hatching (Warkentin 1995, 2000). When to hatch is essentially a behavioral decision, because embryos hatch by vigorous movements that rupture the egg capsule; immobile embryos do not hatch (Warkentin 1995). *Agalychnis callidryas* eggs are susceptible to infection by at least one lethal fungus (Villa 1979). Although hatching in response to pathogens could be advantageous, fungi do not provide the vibratory cues that appear to stimulate early expression of hatching behavior in predation events. Here we establish the presence of a fungal pathogen of *A. callidryas* eggs, assess the level of mortality that it causes, and test whether fungal infection induces early hatching. We also infer the phylogenetic position of the fungus from its nuclear ribosomal small-subunit RNA nucleotide sequence.

Methods

All frog eggs and fungus samples were collected from Ocelot Pond, 2 km south of Gamboa in Soberanía National Park, Republic of Panama, from June to August, 1998, under permits from the Panamanian Institute of Natural Renewable Resources.

Testing pathogenicity: inoculation experiments and fungus culture

Several different fungi were observed growing on *Agalychnis callidryas* eggs. To assess which were potential pathogens, we performed preliminary inoculation trials. We transferred small samples of each morphologically distinct fungus from naturally infected eggs onto a few healthy young clutches and monitored fungal growth. Fungi that failed to grow on live eggs were not examined further.

The single species of fungus (Phaeosphaeriaceae: Dothideales) that grew well on live eggs in preliminary trials was used in two inoculation experiments. The first experiment used fungal inoculum from a naturally infected, fungus-killed clutch. The fungus donor clutch and young, apparently healthy clutches were collected 7 June. Pairs of healthy clutches were matched for age (newly laid, $N = 7$ pairs; 1-d-old, $N = 5$ pairs), egg size, and color. Any dead eggs (possibly unfertilized) were carefully removed from the clutches, and experimental (inoculation) and control treatments were randomly assigned within pairs. A small clump of mycelium was transferred from the donor clutch to each experimental clutch at 2030. Each clutch was hung over rainwater in an individual plastic cup, placed in a larger plastic tub covered with screening to exclude insects and other potential egg predators or parasites, and held in the shade in an open-air laboratory. Eggs were misted occasionally with rainwater to prevent desiccation. Clutches were checked daily for evidence of fungal growth and egg mortality.

In the second experiment, 10 randomly chosen, healthy, newly laid clutches were infected with inoculum from pure cultures of the fungus. Pure cultures were obtained from naturally infected clutches by placing mycelium plucked from the eggs onto potato dextrose agar (PDA; Difco, Detroit, Michigan, USA) with 50 μg/mL each of penicillin G and streptomycin sulfate (Sigma, St. Louis, Missouri, USA) to control bacterial growth. Experimental clutches and matched controls were collected on 10 August, and experimental clutches were inoculated at 2000 h. Paired inoculated and control clutches were grouped together in tubs, and clutches were maintained and monitored as previously described. To confirm that the fungus growing on infected clutches was the same as that experimentally inoculated on these clutches, thus fulfilling Koch's postulates (Agrios 1988), we isolated pure cultures from each successfully infected clutch and compared the fungal morphology to confirm species identity.

Fungal prevalence, mortality, and hatching rates

The prevalence of the fungus on *A. callidryas* egg clutches was assessed in two ways. As part of a study on egg predation (Warkentin 2000), 123 individually marked egg clutches were monitored daily at Ocelot Pond from the morning after oviposition until hatching (following Warkentin 1995). Clutches were monitored in two series, 10–16 June and 19–27 July 1998 (52 and 71 clutches, respectively). Fungal growth on clutches was identified by the presence of mycelium visible to the naked eye. Because not all fungi growing on eggs are pathogenic, and the different species were not initially distinguished in the field, egg mortality was assigned to the presence of fungus only if no other cause of mortality (e.g., wasp predation or desiccation) was evident.

As a second measure of prevalence, 121 young

clutches (≤2 d old) were collected from the pond before it was evident whether they were infected with fungus or not, and then were reared in a protected environment. The clutches were collected on five dates, from 30 May to 24 June 1998, and collections ranged from 12 to 34 clutches each. Eggs in each clutch were counted, and any dead or undeveloped eggs (possibly unfertilized) were noted. Clutches were maintained and monitored as previously described. Fungus growing only on dead eggs was distinguished from fungus growing on and killing live eggs.

To assess the effect of the fungus on both egg mortality and hatching time, each infected clutch was paired with a fungus-free clutch of the same age collected on the same date, as a control ($N = 8$ pairs). Infected and control clutches were monitored daily for egg mortality and, once hatching began, several times daily (on average, every 5 h) for tadpole hatching. Because clutches were checked for hatching at different times among the five clutch series, for graphical presentation of data, the proportion of eggs hatched in each clutch at standardized times was estimated, if necessary, by linear interpolation between actual counts. To further assess the effect of the fungus on the timing of hatching, egg clutches inoculated with fungus in the pathogenicity experiments and their matched healthy control clutches were similarly monitored for hatching time.

To test if the fungus is associated with tadpoles hatching from infected clutches, or if the tadpoles escape uninfected, we attempted to isolate the pathogen from 10 tadpoles hatched from each of 10 infected clutches. We inoculated 10 healthy 1-d-old clutches with fungus from pure culture on 20 August and maintained these clutches as previously described. At age 5 d, we mechanically induced hatching and collected hatchlings, including both those emerging from eggs in contact with the fungus and some from more distant eggs. Hatchlings were rinsed with sterile water to remove potential inoculum that would be readily washed off by pond water after hatching. After being rinsed, tadpoles were plated on PDA medium with antibacterial antibiotics. Each plate was monitored daily for 14 d and emerging fungi were isolated for identification.

Identification of the fungus

Identification of the fungus occurring on clutches of *A. callidryas* was not possible on PDA medium because no diagnostically informative reproductive structures were produced. We attempted to stimulate sporulation by culturing it on low-nutrient medium, such as water and chitin agar. Also, the remains of naturally infected and inoculated clutches were maintained for several weeks after all embryos had hatched or died, to determine if spores would be produced at this stage. An isolate of the fungus is deposited at the American Type Culture Collection, Manassas, Virginia, USA (accession code MYA-909).

In addition to using morphological characters, we attempted to identify the fungus based on the nucleotide sequence of the nuclear small-subunit ribosomal RNA (nSSU). Mycelium for DNA extractions was produced by inoculating the fungus into liquid potato broth (PDB) and shaking at 100 rpm for ~20 d. DNA extractions followed Rehner and Samuels (1994). The nSSU was amplified with primers NS1 and NS4 and was sequenced with primers NS1, NS2, NS3, and NS4 (White et al. 1990). Cycle sequencing was performed with BigDye Cycle Sequencing kits (Applied Biosystems Institute [ABI], Foster City, California, USA) and the sequence data collected on an ABI 377 automated sequencer. Sequences were edited and joined using Sequencher software (Gene Codes; Ann Arbor, Michigan, USA). Fungi with the most similar nSSU sequence to our isolate were identified using the BLAST search option in GenBank (National Center for Biotechnology Information, Bethesda, Maryland, USA).

RESULTS

Pathogenicity and mortality rate in infected clutches

One species of fungus readily grew on live, healthy eggs and killed them. This fungus grew well on PDA, and mycelium transferred from pure culture to healthy eggs resulted in infections. In all cases, the fungus reisolated from experimentally inoculated egg clutches was morphologically indistinguishable from the culture that provided the original inoculum.

Fungal growth in inoculated clutches was not evident to the naked eye until 1.5–2 d after inoculation. Likewise, natural infections were not obvious until >1 d after collection. As infections progressed, fungal growth and the rate at which it killed eggs accelerated. Once the fungus was well established on a clutch, it grew over and killed newly contacted eggs within a few hours.

Mortality in infected clutches was higher than in control clutches (Fig. 1; Friedman tests: inoculated from pure culture, test statistic = 9, $N = 9$, $P = 0.0027$; inoculated from eggs, test statistic = 11, $N = 11$, $P = 0.0009$; naturally infected, test statistic = 8, $N = 8$, $P = 0.0047$). The fungus failed to grow in one clutch inoculated from naturally infected eggs and in one clutch inoculated from pure culture; therefore, these pairs were not included in the analyses. Control clutches showed no sign of fungal growth. Egg mortality in the control clutches (Fig. 1) was largely due to developmental abnormalities and possibly to oxygen stress in the case of eggs completely buried under other eggs. One control clutch had unusually high (24%) mortality associated with a slimy appearance of dead eggs, perhaps due to infection by a different, apparently nonfungal, pathogen. As in control clutches, a few eggs died in fungus-infected clutches from nonfungal caus-

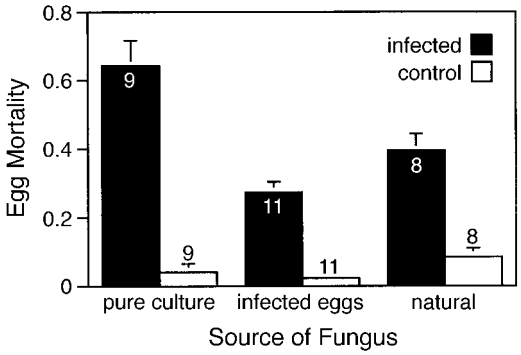

FIG. 1. The proportion of embryonic mortality (mean + 1 SE) in clutches of *Agalychnis callidryas* infected with fungus and in matched control clutches reared in a protected laboratory environment. Infected clutches were inoculated with fungus from pure culture, inoculated from infected eggs collected from a natural pond, or naturally infected prior to collection. Sample sizes are indicated.

es, usually after showing developmental abnormalities. However, most mortality in fungus-infected clutches was clearly caused by the fungus. Fungal hyphae grew over and into live eggs, penetrating and enveloping the developing embryos (Fig. 2). Fungus killed 20.9 ± 3.5 eggs (mean ± 1 SE) in naturally infected clutches (N = 8), 12.3 ± 1.3 eggs in clutches inoculated from other eggs (N = 11), and 27.1 ± 3.6 eggs in clutches inoculated from pure culture (N = 9).

Natural infection rate

Embryos were unambiguously killed by fungus only in two of 123 clutches (1.6%) monitored at Ocelot Pond, Panama; 94 clutches suffered wasp or snake predation, drowning, or desiccation (Warkentin 2000). In some cases, these clutches then contained dead eggs, which often support saprophytic fungi, making it difficult to identify any additional pathogenic fungal growth under field conditions. In other cases (i.e., snake predation), the entire clutch was consumed too soon for any possible fungal infection to become visible. Among the remaining 29 clutches, egg mortality was clearly caused by fungus in two (6.9%).

Of the clutches collected from Ocelot Pond, 8/121 (6.6%) were infected with the pathogenic fungus. Infection rates ranged from 0% to 15.4% in the different collections, averaging 7.8 ± 2.5% (N = 5 collections).

Hatching pattern of infected and healthy clutches

Clutches infected with fungus hatched earlier than did healthy control clutches (Fig. 3). In both infected and healthy clutches, hatching was gradual and asynchronous, but infected clutches started hatching earlier and the whole hatching process was accelerated by up to 1 d compared to controls (Table 1). We compared the midpoint of hatching (50% of live eggs hatched) in infected clutches vs. their matched controls. In all cases, it was significantly earlier (Friedman tests: inoculated from pure culture, test statistic = 4.5, N = 8,

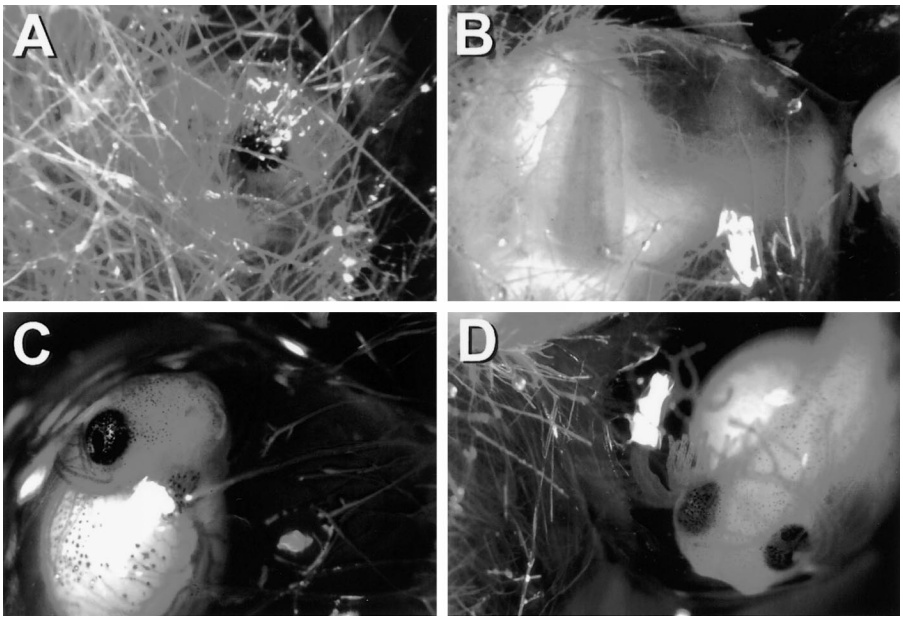

FIG. 2. Fungus growing on *Agalychnis callidryas* eggs. (A) A four-day-old embryo killed by fungus, showing substantial fungal growth on the outside of the capsule, as well as inside it. (B) Young (2-d-old) embryo killed by fungus. Note that much of the mycelium is growing on the embryo itself, within the egg capsule. (C) Fungal mycelium beginning to grow on an egg newly capable of hatching (4-d-old). The embryo is likely to escape the fungus by hatching early. (D) Fungus beginning to grow on 3-d-old egg. The embryo is alive and has not yet been trapped by hyphae, but it is too young to hatch and therefore is likely to die.

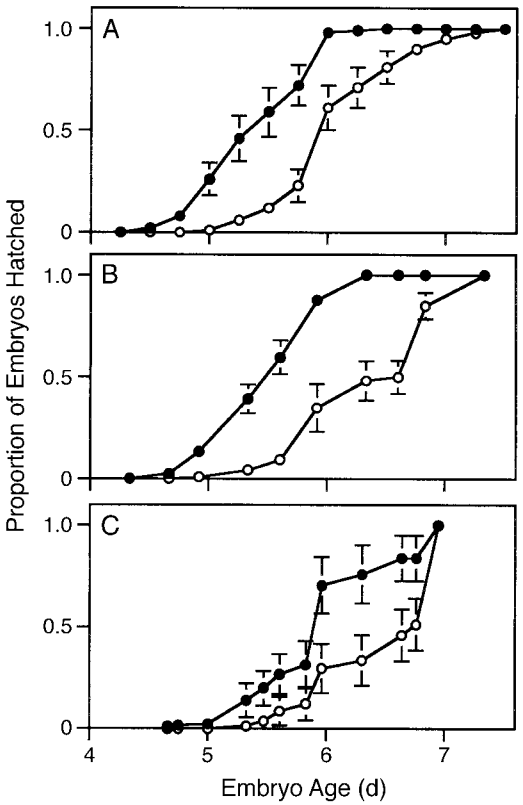

FIG. 3. Hatching pattern of *Agalychnis callidryas* eggs infected with lethal fungus (solid circles) and healthy controls (open circles). Clutches were (A) naturally infected with fungus, (B) inoculated from naturally infected eggs, and (C) inoculated from pure culture. Data are the mean (±1 SE) proportion of embryos hatched at each age, of all embryos that eventually hatched. Sample sizes were: naturally infected clutches, $N = 8$; inoculated from eggs, $N = 11$; inoculated from pure culture, $N = 9$ control, $N = 8$ experimental. The fungus killed all eggs in one clutch inoculated from pure culture. Age of the embryos was measured from midnight on the night of oviposition.

$P = 0.03$; inoculated from eggs, test statistic = 9, $N = 11$, $P = 0.003$; naturally infected, test statistic = 4.5, $N = 8$, $P = 0.03$).

Eggs in healthy clutches showed no obvious spatial pattern of hatching; embryos hatched in an apparently random order from all areas of the clutch. In contrast, the first eggs to hatch in fungus-infected clutches were, with only one exception, in direct contact with the fungus, although they were not completely overgrown. Often the eggs that hatched first left an empty, embryo-free border around the fungus, which the mycelium did not appear to cross (Fig. 4). Eggs physically separated from the fungus in this way were never killed, and hatched later than eggs in direct contact with the fungus.

Fungal identity and transmission

The fungus grew readily on nutrient-rich medium (PDA) and on healthy *A. callidryas* eggs, but did not sporulate. It also failed to sporulate on nutrient-poor medium, on which it grew poorly. Fungus growing on eggs did not produce reproductive structures, even after the embryos had hatched or died. Healthy clutches maintained in close proximity to fungus-infected clutches (as little as 2 cm away) in the laboratory did not become infected with fungus. We were also unable to culture the fungus from any tadpoles hatching out of infected clutches.

A GenBank BLAST search using the nSSU sequence of the fungus (GenBank accession number AF237611) to query public access DNA databases revealed that the sequence most similar (differing by 9 bp (base pairs); 0.8%) was that of *Montagnula opulenta* (Dothideales: Phaeosphaeriaceae). The next most similar sequence (17 bp; 1.5%) was *Phaeodothis winteri*, also classified in the family Phaeosphaeriaceae.

DISCUSSION

Pathogenic fungal infection of eggs

Eggs of *Agalychnis callidryas* host a fungal pathogen in the family Phaeosphaeriaceae (Dothideales: Asco-

FIG. 4. A 6-d-old *Agalychnis callidryas* clutch infected with lethal fungus at Ocelot Pond. The fungus has killed at least 20 embryos. Several embryos have hatched from eggs that were in contact with the fungus, leaving a clear space between the dead, fungus-covered eggs and the remaining 10 live embryos.

TABLE 1. Acceleration of hatching of *Agalychnis callidryas* egg clutches by a lethal fungus. Infected clutches reached defined points in the hatching process earlier than did matched healthy control clutches.

	Difference between infected and healthy clutches (h)					
	Initiation of hatching		50% hatched		Finished hatching	
Source of fungus	$\bar{X} \pm 1$ SE	N	$\bar{X} \pm 1$ SE	N	$\bar{X} \pm 1$ SE	N
Natural infection	13.4 ± 5.0	8	14.5 ± 3.9	8	19.7 ± 4.5	8
Inoculated from infected eggs	23.4 ± 3.6	11	20.5 ± 3.6	11	17.2 ± 4.5	11
Inoculated from pure culture†	13.8 ± 4.8	8	13.3 ± 4.2	8	11.4 ± 4.4	8

† In one clutch inoculated from pure culture, no embryos survived to hatch.

mycota). Samples isolated from diseased clutches into pure culture cause new infections when transferred to healthy clutches, and the pathogen is recoverable from these inoculated clutches, fulfilling Koch's postulates of pathogenicity (Agrios 1988). Mortality of arboreal frog embryos in association with fungal infection has been described (Villa 1979), but the pathogen was not successfully isolated and re-infected into healthy eggs.

We observed no spore-producing structures in this fungus, either in pure culture or on eggs in the field or laboratory. This lack of reproductive structures limits our ability to identify the fungus based on morphology. Phaeosphaeriaceae is poorly characterized taxonomically and, in the absence of comprehensive systematic studies of the group, our molecular data allow identification only to the family level. Fungi in this family have a cosmopolitan distribution and are known to be pathogens of plants or other fungi.

Wind dispersal of the egg-killing fungus seems unlikely, as it apparently does not produce spores in nature, at least not in association with clutches of frog eggs. This conclusion is further supported by our observation that uninfected eggs in close spatial association with infected clutches do not become infected. Thus it appears that the fungus is transported between egg clutches by a vector. Egg clutches of *A. callidryas* and other leaf-breeding frogs are host to larvae of several species of flies, which may act as vectors for moving the fungus between clutches (Villa 1979). These include embryo predators, scavengers that feed on dead eggs, and some which eat egg-infecting fungal mycelium (Villa 1977, 1980, Villa and Townsend 1983, Vonesh 2000; K. M. Warkentin, *personal observation*). Indeed, the egg predator *Hirtodrosophila batracida* (Drosophilidae) is a member of a genus in which all other species are mycophagous (Courtney et al. 1990, Grimaldi 1994); the ancestors of these flies may have fed on egg-infecting fungus. Egg-eating wasps might also transport fungal hyphae from clutch to clutch as they forage.

Only a small proportion of the total mortality of *A. callidryas* eggs at Ocelot Pond in Panama was unambiguously caused by fungal infection, much less than that caused by egg predators. Infections were more frequently detected in clutches protected from predators (6.6% infected), suggesting that the initial rate of fungal infections may be higher, but that some infected clutches are damaged or eaten by predators before substantial fungal growth occurs. This estimate of initial infection rates is a minimum value, because clutches were also protected from potential sources of infection after their collection from the pond. Lethal fungal infections of *A. callidryas* clutches occur at low frequencies in Corcovado National Park and at La Selva Biological Station, Costa Rica (K. M. Warkentin, *unpublished data*). Villa (1979) described a morphologically similar pathogenic fungus growing on the arboreal eggs of three anuran species. He reported maximum infection rates of 10% for *A. callidryas* in Matagalpa, Nicaragua, and 25% for *A. annae* near San José, Costa Rica. *Hyalinobatrachium fleishmanni* eggs, which are paternally attended, were rarely infected (Villa 1979). For comparison, Kam et al. (1996) found a fungal infection rate of 5.4% for the arboreal eggs of *Chirixalus eiffingeri*, a hole-nesting species without egg brooding, and Simon (1983) found a 70% infection rate for unbrooded terrestrial eggs in *Cophixalus parkeri*, a typically egg-brooding species.

Within *A. callidryas* egg clutches infected by the fungus, mortality can be substantial, but is rarely 100%. In this study, fungus killed, on average, 40% of eggs in naturally infected clutches; and in only one clutch inoculated from pure culture were all eggs killed. This contrasts strongly with Villa's (1979) fungus, which completely overgrew clutches of up to 100 embryos in under 48 h, causing 100% mortality unless infection occurred late in development. Villa's (1979) species was not deposited in a culture collection; thus a taxonomic comparison between these fungi is not possible.

Embryonic response to fungus

Adaptive behavioral responses of animals to pathogens are widely hypothesized, but have been clearly demonstrated in only a few host species (Poulin 1995, Kiesecker et al. 1999). *Agalychnis callidryas* behaviorally accelerates hatching in response to infection of egg clutches by a lethal fungus. Hatching starts half a day to a day earlier in infected clutches than in uninfected controls, and the entire hatching process is advanced compared with controls. There is also a clear

spatial pattern of hatching in infected clutches; live eggs in direct contact with the fungus hatch first and eggs farther away hatch later. Advanced hatching in infected clutches, especially by the embryos at highest risk, reduces direct mortality from fungal infection. In clutches infected at a young age, by the time embryos become capable of hatching, the fungus is growing rapidly and the interval between first hyphal contact and embryonic death is short (hours; K. M. Warkentin, *personal observation*). Thus embryos already in contact with the fungus when they become competent to hatch would almost certainly die if they were to remain in the egg even half a day longer. Furthermore, if the path of fungal growth was not obstructed by gaps left by hatched embryos, the fungus could contact and kill additional embryos, or induce their early hatching. Thus fungus-induced hatching clearly confers an immediate benefit.

Nonetheless, early hatching has a cost. Embryonic development is remarkably consistent in *A. callidryas*; i.e., age and developmental stage are very tightly correlated within a population and there is no indication that risk to embryos accelerates development (Warkentin 1995, 1999*a*; K. M. Warkentin, *personal observation*). Variation in hatching age therefore creates variation in the developmental stage at which tadpoles enter the water. Embryos hatching early from fungus-infected clutches are less developed than those hatching later from healthy clutches. Less developed hatchlings are more vulnerable to aquatic predators than are more developed, older hatchlings (Warkentin 1995, 1999*b*). Thus the fungus has both a direct effect on embryonic mortality and an effect on hatching stage, which affects the risk of mortality after hatching. Embryos separated from the fungus by gaps left by eggs that hatched early are able to avoid this cost by hatching relatively late, many within the range of hatching of uninfected eggs (Fig. 3).

Despite its cost, early hatching may be the best or only antifungal defense available to embryos. The two potential alternatives are chemical and immunological defenses. Antimicrobial peptides in the skin are an important defense of adult amphibians against pathogens, including fungi (Mor et al. 1994, Goraya et al. 2000). Some defensive peptides are first produced at metamorphosis (Clark et al. 1994, Reilly et al. 1994), whereas others are found in tadpoles but have not been detected in embryos (Wabnitz et al. 1998). Similarly, amphibian embryos are tolerant of foreign antigens; immune responses first appear some time after hatching, and improve ontogenetically (Harris 1941, Hildemann 1966, Volpe 1980, Du Pasquier et al. 1996). Thus embryos appear unable to defend themselves against pathogens immunologically or chemically. Furthermore, because fungi establish first on the egg capsule, they may smother embryos before penetrating their bodies. This would protect them from any early-developing skin peptides or immune responses. Fungi could be vulnerable to maternally produced toxins in the egg capsule; however, these would probably carry fixed costs to the female and to the embryo (Orians and Janzen 1974), regardless of the presence of the pathogen.

The pattern of accelerated hatching in fungus-infected clutches is different from early hatching in clutches attacked by snakes or wasps. Clutches infected with fungus hatch gradually over a period of days, but the hatching curve is shifted to a younger age than in healthy clutches. In snake attacks, except for the eggs that are eaten, entire clutches hatch in minutes (Warkentin 1995). In wasp attacks, the individual eggs under direct attack, and some neighboring eggs, hatch immediately (Warkentin 2000). The cues initiating early hatching are also different in fungus infections than in snake and wasp attacks. The critical element of predator attack that induces immediate hatching appears to be physical disturbance, i.e., movement of the eggs. Artificially jiggling eggs also stimulates immediate hatching (Warkentin 1995). Fungal growth into eggs, however, does not move them. This lack of movement may account for the asynchrony in fungus-induced hatching.

There are three likely, and not mutually exclusive, mechanisms by which fungus could stimulate early hatching. First, embryos might respond to a chemical released either by the fungus itself or by other embryos being killed. Other amphibian embryos respond to chemical cues from predators or predation events by delaying hatching (*Ambystoma barbouri;* Sih and Moore 1993, Moore et al. 1996) or accelerating hatching (*Hyla regilla* and *Rana cascade*; Chivers et al. 2001). Second, the fungus could reduce oxygen availability to embryos either by reducing the exposed egg surface area available for oxygen diffusion, or by directly competing with embryos for oxygen within egg capsules. In several species of amphibians and fish, oxygen stress accelerates or induces hatching (DiMichele and Taylor 1980, Petranka et al. 1982, Bradford and Seymour 1988). Once capable of hatching, *Agalychnis callidryas* eggs hatch rapidly if exposed to hypoxic gas mixtures or if submerged in water, which reduces oxygen diffusion (K. M. Warkentin, *unpublished data*). Third, the fungus may induce hatching by physically irritating embryos. *Agalychnis callidryas* embryos frequently move within their egg capsules, and fungal hyphae growing into the perivitelline space obstruct these movements. Physical contact with the hyphae may irritate embryos and stimulate the more vigorous movements that cause hatching.

Evolution of embryonic defenses

Adaptive responses of post-hatching animals to predators are common, and include morphological changes, life history shifts, and behavioral responses (Havel 1987, Lima and Dill 1989, Harvell 1990). Adaptive responses to pathogens are also known, although phys-

iological responses have been better demonstrated than behavioral ones (Ewald 1994, Poulin 1995, Atlas and Bartha 1998, Kiesecker et al. 1999). Embryos face multiple risks and often high mortality levels, and appropriate responses to these risks should be favored by selection, as in later life stages. Nonetheless, embryonic antipredator and antipathogen defenses have received little attention until recently.

Predator effects on hatching stage have now been documented in six species of amphibians (Sih and Moore 1993, Warkentin 1995, Vonesh 2000, Chivers et al. 2001; G. Schalk, *personal communication*), and have been suggested in crustaceans (Blaustein 1997). Pathogen infection of eggs accelerates hatching not only in *A. callidryas,* but also in *Rana cascadae, R. clamitans, Bufo boreas,* and *Hyla versicolor* (J. Kiesecker, *personal communication*). Further investigation is likely to reveal additional cases of embryonic antipredator and antipathogen responses.

Knowing that some embryos respond to mortality risks by altering their hatching stage, an important question is: what determines which species will respond to which risks? An ecological trade-off between pre- and post-hatching risks creates conditions in which phenotypic plasticity in hatching can be favored. The combination of cues provided by the source of risk and the nature of the hatching process may constrain when hatching plasticity can be an effective defense. Hatching in amphibians and fish is often a largely enzymatic process. Developing embryos synthesize proteolytic enzymes that digest the egg capsule, or components thereof, allowing the embryo to escape (Carroll and Hedrick 1974, Schoots et al. 1982). Although in some fish the process of choriolysis and hatching can be rapid (e.g., 10–30 min in *Fundulus heteroclitus*; DiMichele et al. 1981), in anurans studied to date, the breakdown of the egg capsule prior to hatching appears to be more gradual (e.g., Yoshizaki 1978, Yamasaki et al. 1990, Yoshizaki and Yamasaki 1991). The essentially instantaneous, behavioral hatching response of *A. callidryas* in predator attacks (Warkentin 1995, 2000) is dramatically faster than the enzymatic processes previously described in anamniote vertebrates, suggesting that mechanisms of hatching vary among species. Similarly rapid, behaviorally mediated hatching in response to physical disturbance has recently been described in a fish (*Leuresthes tenuis;* Griem and Martin 2000) and in another frog (*Rana arathrooni;* Brown and Iskandar 2000).

For early hatching to be an effective defense against a source of egg mortality, it must be deployed in time for embryos to escape. If predator cues are only available upon attack, as is likely for arboreal and terrestrial eggs, defensive hatching may be restricted to species capable of rapid, behaviorally mediated escape from the egg. If cues from egg predators are available in the absence of direct attack, for instance in aquatic eggs bathed by waterborne chemical cues, species with more gradual hatching could also benefit from plastic, predator-accelerated hatching. When pathogens or micropredators such as fly larvae develop in egg clutches, the source of mortality is continuously present providing cues, but it kills embryos gradually over an extended period (Villa 1980, Villa and Townsend 1983, Vonesh 2000). In these cases, there is time for physiological or developmental, as well as behavioral, responses to the risk. If the mechanism of hatching is more constrained than the stimuli that trigger it, the set of species that could evolve pathogen-induced early hatching is broader than the set that could evolve predator-induced early hatching.

Life history theory suggests, and empirical studies have found, that the timing of transitions between life stages is sensitive to the mortality risk costs and the growth benefits that accrue in each stage (Werner and Gilliam 1984, Werner 1986, 1988, Skelly and Werner 1990). Pathogens can reduce host growth rates, directly kill hosts, and make hosts more susceptible to other causes of mortality (Dobson and Hudson 1986, Price et al. 1986, Dobson and Crawley 1994). As with fungal infection of *A. callidryas* eggs, these costs can be stage specific (e.g., Kiesecker and Blaustein 1999). Where pathogen pressure differs between any two adjacent life stages, it should influence the timing of the transition between them. Studies of pathogen effects on other transition points, such as metamorphosis, that are known to respond adaptively to environmental variation would clarify the general importance of pathogens in shaping life histories.

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