Dermomyces sp. Infection at Different Stages of Ranid Development

By

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Chapter 1 – The Problem of a Novel Pathogen

Disease is among the many synergistic factors contributing to global amphibian population decline (Blaustein & Bancroft, 2007). Particular natural history traits of amphibians could facilitate more frequent infection (Blaustein & Bancroft, 2007). Amphibians inhabit both terrestrial and aquatic environments. This dual life style exposes them to a greater diversity of disease agents (Blaustein & Bancroft, 2007) in comparison with organisms restricted to one particular environment. *Dermomyces* sp. is a parasite that specializes in anuran hosts. It is a novel agent of particular concern because it has cause mass mortalities of anuran larvae, including the offspring of the dusky gopher frog, *Rana sevosa* (Cook, 2008).

There are two overarching aqueous anuran life stages, the embryo and larva. The embryonic stage includes all development between fertilization and hatching, while the larval stage encompasses development after the embryo has hatched and before it becomes fully terrestrial. In the natural environment, the aquatic life stages of anurans are at the greatest risk of infection by *Dermomyces* sp. (Cook, 2008; Green et al., 2003). However, Cook (2008) encountered an adult *R. sevosa* approximately three years of age with *Dermomyces* sp. spores in its intestine. He also successfully infected an adult *R. catesbeianna* by intraperitoneal injection.

Infected hosts can contain spores restricted to the lumen of the intestine and possibly at some primary infection site. Infection does not always lead to disease, and spores in the lumen alone have not been observed to be fatal (Cook, 2008). Disease occurs in embryos or larvae that have a severe infection with large numbers of spores in organs and tissues, and is usually indicated by an enlarged liver overridden by spores and consisting of little functional liver tissue (Cook, 2008). Green (et al., 2003) observed that the liver, spleen, pronephroi, and mesonephori
of severely infected larvae were enlarged and “homogeneously white” in appearance. Cook (2008) also notes that disease creates an enlarged, whitened liver.

*Dermomyces* sp. infection has been reported in Louisiana, Mississippi, Alabama, Florida, (Cook, 2008), Maine (Gahl, 2007), Georgia (Davis et al., 2007), Alaska, Minnesota, Virginia, and North Carolina (Green et al., 2003). Cook’s (2008) findings indicate that anurans in the genus *Rana* are more susceptible to infection, and he notes that this is the only genus that has suffered mass mortalities. Infected ranids include *R. capito*, *R. sphenophala*, *R. gryllio*, *R. clamitans*, *R. catesbieana*, *R. sylvatica*, *R. septentrionalis*, and *R. sevosa* (Cook, 2008; Davis et al., 2007; Green et al., 2003). Infection has also been reported in *Gastrophryne carolinensis*, *Psuedacris ornata*, *Acris gryllus*, *Hyla cineria*, *H. gratiosa*, and *H. femoralis* (Cook, 2008). Mass mortalities have occurred in Florida, Mississippi, Georgia, Virginia, New Hampshire, and Minnesota (Cook, 2008; Green et al., 2003; Davis et al., 2007). Mass mortalities of *R. sphenophala* have taken place in Mississippi, Georgia, and Florida (Davis et al., 2007; Cook, 2008), and mass mortalities of *R. capito*, *R. catesbeianna*, and *R. sevosa* have occurred in Mississippi and Florida (Cook, 2008).

Of the above mentioned anuran species, the Fish and Wildlife Services listed *R. sevosa* as endangered in December 2001 (LaClaire, 2001). The historical range of this species included Louisiana, Mississippi, and Alabama (Goin & Netting, 1940; 1942), but habitat has been severely restricted. We now know of only two *R. sevosa* breeding populations, one in Harrison County and another in Jackson County, Mississippi (Cook, 2008), and a recent estimate is that only 100 adults survive (Cook, 2008). These breeding ponds are essential in maintaining *R. sevosa* populations, yet housing development that will further restrict available habitat is planned near the Harrison County pond (LaClaire, 2001). An added threat to the *R. sevosa* population at
the Harrison County pond is mass mortalities of *R. sevosa* larvae caused by *Dermomyxoides* sp. (Cook, 2008). This situation is made more pressing because *R. sevosa* does not breed every year, and its population cannot afford seasons of low recruitment into the adult population (Richter & Seigel, 2002). Because infection caused by *Dermomyxoides* sp. Can drastically influence the number of anuran larvae that successfully transition into adulthood, this pathogen is a significant threat to the conservation of *R. sevosa*.

While some aspects of infection by *Dermomyxoides* sp. have been examined, the disease ecology of this pathogen is largely unexplored. Disease ecology is the study of the relationships between the disease agent and host(s). This research plans to answer the following three questions as they pertain to the disease ecology of *Dermomyxoides* sp.:

1. At what stage of anuran development can infection by *Dermomyxoides* sp. occur?
2. Is there any relationship between the progression of infection by *Dermomyxoides* sp. and the development of particular organs and/or tissues in the anuran host?
3. Does the initial stage of exposure to *Dermomyxoides* sp. affect infection?

**Chapter 2 - Literature Review: Background Information & Justification of Questions**

The phylogeny of this agent is disputed. *Dermomyxoides* sp. belongs in the order Dermocystida and falls into the “catchall group” of dinoflagellates within the alveolates (Robin Overstreet, personal communication). Davis (et al., 2007) concluded from the sequencing of rRNA that the clade into which *Dermomyxoides* sp. fits is sister to the clade containing marine pathogens of the genus *Perkinsus*. Phylogeny aside, the physical dimensions of two life phases of *Dermomyxoides* sp. are known, and identification of this pathogen in histological sections is possible.
The spore and zoospore life phases have been identified, the latter of which has been described solely by Cook (2008). Davis (et al., 2007) recorded organisms of *Dermomycoides* sp. with a mean diameter of 6.2 μm. Cook (2008) found that spores had a mean diameter of 6.3 μm and a mean breadth of 6.0 μm. Spores of *Dermomycoides* sp. are spheroid in shape (Cook, 2008; Davis et al., 2007). Using scanning electron microscopy, Davis (et al., 2007) found that simple polyhedrals covered the surface of the spore. While this phase is a resting life phase of *Dermomycoides* sp., zoospores are a motile. Zoospores have a mean length of 3.6 μm, an average breadth of 3.3 μm, and a body shape that is almost spherical (Cook, 2008). They have a single flagellum with a mean length of 4.5 μm (Cook, 2008). While the zoospore phase follows from the rupturing of the spore, it is uncertain whether there are discrete life phases between these two phases.

Both the spore and the zoospore life phases of *Dermomycoides* sp. are capable of infecting anuran offspring, however they do so differently. Spores are incapable of infecting anuran embryos, as infection via spores requires that they be ingested. For ingestion to occur, larvae must be able to feed (Gosner stage 25) (Gosner, 1960), and the teeth should be fully formed (Shumway, 1940). In the wild, infection has been observed just before tooth development is complete (Gosner stage 24), through metamorphosis (Gosner stage 46), and into adulthood (Cook, 2008). The presence of infection prior to the complete development of the larval teeth (Gosner stage 24) has two plausible explanations. Either fully formed teeth are not required for larval feeding, or zoospores caused earlier infection and spores of *Dermomycoides* sp. formed within the body. Zoospores of *Dermomycoides* sp. infect by penetrating host tissues (Cook, 2008). If zoospores are the life phase carrying out initial infection, then infection can feasibly
occur at any point in anuran development (Robin Overstreet, personal communication). We do not presently have transition electron microscopy (TEM) imagery of penetration by zoospores.

In controlled experiments where anuran embryos were exposed to zoospores of *Dermomycoïdes* sp., Cook (2008) identified spores as early in anuran development as mid-nerulation (Gosner stage 15), during the formation of what will become the dorsal, hollow nerve cord (Gosner, 1960; Shumway, 1940). This finding indicates that embryos were penetrated by *Dermomycoïdes* sp. prior to mid-nerulation (Gosner stage 15), but evidence of this has not been found. The earliest stage at which anurans become susceptible is unknown, and it is a query that this research hopes to answer. Wildlife management officials would benefit from the knowledge of exactly when infection by *Dermomycoïdes* sp. can set in. That knowledge would give them the opportunity to remove egg masses before they become infected and raise those anuran offspring in the laboratory setting.

The transition from spore to zoospore is influenced by external environmental conditions. It is necessary for wildlife managers to monitor these conditions, as anuran embryos and larvae can survive infection by spores, but zoospores usually cause mortality (Cook, 2008; Robin Overstreet, personal communication). Zoospore density increases if the spores are desiccated prior to hatching (Cook, 2008). For anurans that breed in ephemeral or perennial ponds, like *R. sevosa* (Jensen et al., 2003; LaClaire, 2001; Richter & Seigel, 2002), penetration by zoospores of *Dermomycoïdes* sp. is highly likely because the spores are frequently exposed to dry conditions (Cook, 2008). Caution must be used when attempting to control the zoospore population by artificial extension of the hydroperiod of a pond. While the addition of water provides anuran larvae with more time for metamorphosis and has been successfully used to in conservation efforts of *R. sevosa* (Siegel et al., 2006), the added water can cause the pH level of the pond to
change. The number of zoospores hatched from spores of *Dermomyxoides* sp. could increase depending on the direction and magnitude of that pH change.

Zoospores hatch more readily at a pH of 6.5 (Cook 2008). This pH can come into effect after the addition of a significant amount of water to a pond, after a heavy rain, for example. The Nature Conservancy (TNC) Pond in Mississippi is a translocation pond for *R. sevosa*. After six different rain events, TNC Pond approached a pH of 6.5 (Cook, 2008). *R. sevosa* breeding is tightly correlated with heavy rains (Richter & Seigel, 2002). This natural history trait may expose *R. sevosa* to waters with pH values closer to that value preferred for hatching of zoospores of *Dermomyxoides* sp. Like the pond in Harrison County, TNC Pond has produced infected *R. sevosa* larvae (Cook, 2008). Low canopy cover is also associated with the presence of infection by *Dermomyxoides* sp. (Gahl, 2007).

Biotic factors could potentially contribute to infection by *Dermomyxoides* sp. Gahl (2007) did not find that larval density contributed to infection, however Cook (2008) suggests reducing larval density might prevent further infection by *Dermomyxoides* sp. because there would be fewer vectors of the disease. As anuran larvae of sympatric species can potentially serve as vectors of infection (Cook, 2008), high larval density typical of anuran breeding may be detrimental to the success of those larvae. Although the breeding season for the dusky gopher frog is reported to last from December to March (Richter et al., 2003), *R. sevosa* often breeds less consistently and later in that time frame (Carl Qualls, personal communication). It is likely that when mature *R. sevosa* do arrive at the ponds, earlier breeders with more numerous adult populations, like *R. sphenoecephala*, have already oviposited, providing *Dermomyxoides* sp. with the opportunity to infect multiple hosts. Keisecker and Kiesecker and Blaustein (1997) found that late breeders were more susceptible to *Saprolegnia ferax*, a pathogen of amphibian eggs in the
Pacific Northwest. If a similar pattern exists for *Dermomyoides* sp., then *R. sevosa* later breeding habits may put the species at greater risk of contracting infection by *Dermomyoides* sp. The relationship between infection by *Dermomyoides* sp. and breeding period has not been documented; and while egg masses of *R. sphenosephala* were removed from breeding ponds of *R. sevosa* to informally test whether larval density influenced infection rates, the effect on infection was unknown (Robin Overstreet, personal communication). Removal of portions of the larval population in order to control infection remains an untried option for the management of disease caused by *Dermomyoides* sp.

There have been studies examining factors of the external environment that trigger transition between life phases of *Dermomyoides* sp. (Cook, 2008; Gahl, 2007), however, little investigation into the effect(s) of anatomical changes within the anuran host and life phase transitions has taken place. This research will examine whether the development of particular organs and tissues influences the life phases of *Dermomyoides* sp. Because the liver and kidneys, along with the intestine, are consistently the sites of spore aggregation (Cook, 2008; Green et al., 2003), the development of these organs might be significant to the progression of infection of *Dermomyoides* sp. It is possible that life phases of *Dermomyoides* sp., both named and unnamed, rely on these organs (Robin Overstreet, personal communication), and progression of infection is stalled until the organs become well developed. Knowledge of which organs or tissues influence the progression of infection would be useful to wildlife management because it would allow them to remove infected larvae at those stages before infection becomes severe. This would ensure that the mean intensity of infection in individuals remains low, which would hopefully allow more successful metamorphs to emerge from the pond.
If a connection between specific tissue and organ development in the host and the progression of infection exists, then it serves that infection would be influenced by the stage at which the host is exposed to *Dermomyoides* sp. For example, if infection is positively correlated to the development of the Wolffian bodies, the adult anuran kidneys, then exposing the embryo to *Dermomyoides* sp. before these organs have had time to develop might produce an infection with a lower mean intensity. This is the third query that this research will address. Knowledge of how initial exposure affects future infection could allow wildlife management officials to determine which egg masses or groups of larvae will develop more severe infections based upon when they were exposed to *Dermomyoides* sp. If exposure to *Dermomyoides* sp. at hatching (Gosner stage 17) consistently leads to intense infection, then removal of egg masses exposed as hatchlings should maintain a lower mean infection rate of future larval populations.

**Chapter 3: Methods**

This research has the following objectives:

1. to identify the earliest stage of anuran development infected by *Dermomyoides* sp.
2. to determine the phases of infection and whether they are regulated by the development of particular host organs and tissues within the host
3. to determine if the stage of initial exposure influences whether infection occurs, or how infection develops.

*R. sphenosephala* and *R. catesbeiana* will be used as surrogate species for *R. sevosa*. Eggs and larvae were collected and exposed to *Dermomyoides* sp. in previous experiments conducted by Dr. Robin Overstreet, Joshua Cook, and others. Specimens were fixed in a 10% phosphate buffered formalin solution, embedded in paraffin, and sectioned along cross, frontal, and sagittal
planes to a width of four microns. They were stained using hematoxylin and eosin, and mounted on glass slide with cover slip.

To identify the earliest stage of anuran development infected by *Dermomycoides* sp., the developmental stage of the embryo or larva will be determined according to stages laid out by Gosner (1960). The site(s) at which spores of *Dermomycoides* sp. first appear will be identified, and the stage of initial exposure to *Dermomycoides* sp., type of exposure, and time elapsed between exposure and fixing will be noted.

To achieve the second objective, life phases of *Dermomycoides* sp. will be identified and described. The organs and tissues inhabited by each life phase will be recorded. Infected anuran specimens at various developmental stages will be compared, and differences in the location of spores, the rank of mean intensity of infection, and the presence of different life phases of *Dermomycoides* sp. will be noted.

For the final objective, infected anuran specimens exposed at different developmental stages will be compared using specimens fixed during the same, or at very close, developmental stages. Differences in the presence of spores rank of mean intensity of infection, and life phases of *Dermomycoides* sp. observed will be recorded.


