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A MODEL HOST-PATHOGEN SYSTEM FOR STUDYING INFECTIOUS DISEASE DYNAMICS IN AMPHIBIANS: TIGER SALAMANDERS (*AMBYSTOMA TIGRINUM*) AND *AMBYSTOMA TIGRINUM* VIRUS

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Pathogens are among the suspected causes of declining amphibian populations, but studying infectious diseases in small, threatened populations is ethically and experimentally questionable. Progress on understanding amphibian diseases requires model host-pathogen systems with populations large enough for robust experimental designs that do not threaten the amphibian host with extinction. We report on viral genomics, persistence, and host-pathogen dynamics of a model system we are using for studying an amphibian disease: tiger salamanders (*Ambystoma tigrinum*) and *Ambystoma tigrinum* virus (ATV). ATV is a large, cytoplasmic, double-stranded DNA virus that causes systemic infections in individuals and recurrent epidemics in tiger salamander populations in western North America. The ATV genome is now completely sequenced, which is an important step toward understanding viral pathogenesis. Further, because tiger salamanders and the closely related axolotl have a long history as model organisms for developmental genetics, the genetics, development, and physiology of these species are known at levels that can support detailed studies of the host-virus interaction. Salamanders become infected with ATV via direct contact, feeding on infected tissues, and by immersion in water containing virus particles. There is no evidence of long-term persistence of ATV in the environment outside of salamanders: the virus becomes quickly undetectable in pond water and dry mud, and no other syntopic hosts are known. ATV is usually lethal within 2-3 weeks of infection, although some salamanders lose overt symptoms of infection, including papules and lesions, and survive. In one laboratory experiment ATV was re-isolated from 40% of these survivors, which then transmitted the disease to uninfected salamanders. Chronic infections also occur in field populations and appear to be the means by which ATV persists between epidemics. The tiger salamander – ATV system offers us a model for studying the host-pathogen interactions thought to be threatening some amphibian populations with extinction.

Key words: amphibian declines, conservation, ATV, ranaviruses,

INTRODUCTION

The leading explanations for amphibian declines include land use change, exotic species, commercial exploitation, global change, toxins, and pathogens (Collins & Storfer, 2003). Ecologists and conservation biologists are only just beginning to appreciate the pervasive role that pathogens play in structuring wildlife populations and communities. But more to the point relative to amphibian declines, we are just beginning to understand the conditions under which infectious disease can lead to extinction, and there are only a handful of cases illustrating this mechanism. Disease may have caused the extinction of the Australian marsupial wolf early in the 20th century (McCallum & Dobson, 1995), and clearly caused the extinction of the snail *Partula turgida* in 1996, but the latter is a special case. The last individuals of *P. turgida* in the wild were brought into captivity to avoid extinction from predation by an introduced land snail species, but all died from infection by a lethal microsporidean parasite (Cunningham & Daszak, 1998). Hawaiian honeycreepers seem to offer a case of extinction facilitated by pathogens under field conditions. The avian malarial parasite was probably introduced intermittently into Hawaii for thousands of years, and avianpox virus, potentially fatal but slow acting, was introduced with colonists' poultry. Endemic bird populations were unaffected, however, since neither parasite had a vector in the archipelago. Everything changed early in the 19th century when the mosquito *Culex quinquefasciatus* was inadvertently introduced into Hawaii and began transmitting parasites to native birds. Avian malaria has diminished the sizes and ranges of many native honeycreepers, and is the suspected cause of extinction for several species (Warner, 1968; Benning *et al.*, 2002). The infectious disease chytridiomycosis is also thought to have played a key role in the extinctions of frog species in the Americas and Australia. If this proves to be the case, and the lethal amphibian pathogen continues spreading worldwide, amphibian species losses facilitated by disease will be our most compelling example to date of how a pathogen can act globally and rapidly to cause widespread extinctions.

A combination of forces can propel a parasite – the general epidemiological term for any infectious organism – from being apparently benign to becoming

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dangerous (Lafferty & Gerber, 2002). Exotic species may introduce novel parasites into susceptible populations, or a parasite may already be present in a population, but something in the environment (e.g. toxins, climate change, added predation) makes individual hosts more susceptible. Crowding hosts and high population densities also facilitate transmission of parasites. And of course parasites are not static, but evolve in response to their selective environment. Based on theory we would predict that when transmission rates are high more virulent parasites would be favoured (Ebert, 1999). Virulence traits can help the parasite defeat a host's immune system, improving the likelihood of within-host persistence and eventual transmission. In contrast, high virulence may reduce host activity and possibly also longevity, thereby reducing the period of infectiousness and decreasing the likelihood of transmission; highly virulent pathogens may also "burn out" their host populations and become locally extinct (Rand *et al*., 1995).

What tips the balance from host-parasite (H-P) coexistence to host extinction? Amphibians and their pathogens offer ideal, if unfortunate, cases for studying these forces because study species span a continuum from H-P coexistence, to – we suspect – population declines and extinction. An interdisciplinary research program at the intersection of virulence of parasites in amphibian populations, susceptibility of hosts to infection, and population dynamics of host and pathogen is an effective way to study the forces balancing coexistence and extinction because individually and collectively these elements control H-P systems (Fig. 1; Collins *et al.*, 2003).

TIGER SALAMANDER – VIRUS SYSTEM

Model organisms have played a key role in advancing the life sciences (Kohler, 1994), from biomedicine (Koshland, 1988) to evolutionary biology (Kellogg & Shaffer, 1993). Progress on understanding the contribution of diseases to decline and extinction of amphibians will require model H-P systems with populations large enough to support robust experimental designs. For bioethical reasons we do not want to initiate experiments or observations that might threaten with extinction already imperiled amphibian hosts. Several features of its biology suggest that the tiger salamander (*Ambystoma tigrinum*) and one of its viral pathogens have the qualities of a comprehensive model system for studying how diseases, alone or interacting with other factors, affect amphibian population dynamics, perhaps threatening some populations with extinction.

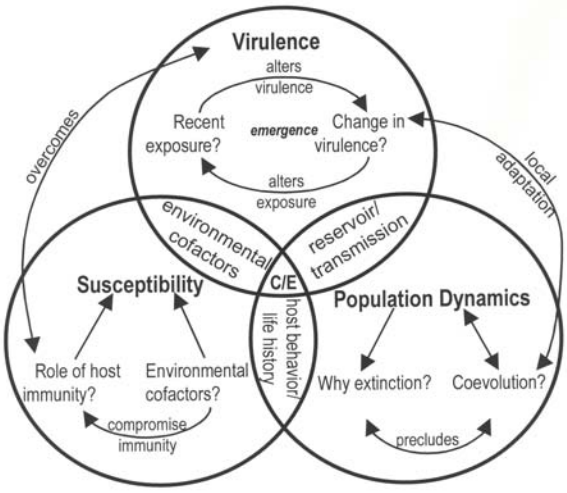

FIG. 1. Venn diagram outlining some of the interactions among virulence, susceptibility, and population dynamics. Ultimately, these three elements of host-pathogen systems determine where species fall on the coexistence-extinction (C/E; center) continuum.

GLOBAL AMPHIBIAN PATHOGENS

Ranaviruses are global amphibian pathogens (Daszak *et al.* 1999; Carey *et al.* 2003; Collins *et al.*, 2003; Daszak *et al.* 2003). Two ranavirus strains were isolated independently from tiger salamander epizootics in North America: *Ambystoma tigrinum* virus from Arizona, USA (Jancovich *et al*., 1997), and Regina ranavirus from Saskatchewan, Canada (Bollinger *et al*., 1999). The two research groups have collaborated in isolating and characterizing ranaviruses from tiger salamander epizootics in six states in the USA, and two Canadian provinces in western North America (Jancovich *et al*., 2003). The isolates had similar genomes, and are now recognized as one widespread species, *Ambystoma tigrinum* virus (ATV) in the genus *Ranavirus* (family *Iridoviridae*).

ATV is a large, cytoplasmic double stranded DNA virus that causes systemic infections. The genome is completely sequenced, and is 106 332 base pairs with 96 putative open reading frames (ORFs) that sort into four functional classes: genes with homology to putative viral/cellular replicative proteins (n=24); genes possibly involved with immune modulation/pathogenesis (n=3); genes with homology to other iridovirus ORFs, but of unknown function (n=61); and genes of unknown function with no homology (n=8) (Jancovich *et al*., 2003). Chromosome 7 of the human genome has nearly 158 million nucleotides of DNA, and some 1917 gene structures (known genes, novel genes, partial genes, predicted genes, putative and noncoding RNA genes) (Scherer, 2003). By comparison, ATV is a much more tractable genome for analysis via functional genomics.

The ATV sequence allows us to do several things. First, functional genomic techniques can yield important clues for fighting disease through an analysis of what all of the genes do. In the recent case of severe acute respiratory syndrome (SARS), the virus sequence allowed researchers to develop the probable structure for a key protein involved in replication, a step in developing a possible drug target (Vogel, 2003). Sequence data can hasten the search for a pathogen's weak spots; in the case of ATV, a starting point would be the three ORFs coding for immune modulation/pathogenesis. We can also use the variability among gene sequences, particularly those involved in pathogenesis, from various

geographic sites to test hypotheses regarding the spatial and temporal spread and emergence of disease; we are doing just that (Jancovich, *et al.* in prep.). Tiger salamanders have a wide geographic range and well-known systematic relationships, making these spatial and temporal analyses robust. Further, tiger salamanders are easily manipulated in the field and laboratory, and occur in large population sizes allowing experimental work on virulence, resistance, and susceptibility in this H-P system.

Having the gene sequence of the host as well as the pathogen would be especially valuable for analyzing interactions between the two species and their co-evolution. *Ambystoma tigrinum* offers the possibility for such a dual analysis of the functional genomics of host and pathogen because of its close relationship to the axolotl, *Ambystoma mexicanum* (Shaffer, 1993). The axolotl has been a leading model in developmental biology throughout much of the 20th century (Armstrong & Malacinski, 1989), making it the object of an extraordinary amount of research (Smith & Smith, 1971). Furthermore, we now know that axolotls carry lethal ranaviruses closely related to ATV (Davidson *et al.*, 2003). Voss *et al.* (2001) used genetic linkage analysis to identify chromosome segments homologous between ambystomatid salamanders and distantly related vertebrate models. Their study animals were the axolotl and *Ambystoma tigrinum tigrinum* because of their long history as research models. They concluded that, "comparative gene mapping appears to be an efficient strategy for identifying orthologous loci between ambystomatid salamanders and genomically well characterized vertebrate model organisms." There is a distinctive opportunity for studying the H-P biology of an infectious disease in amphibians by uniting the functional genomics of ATV with the bridge between *Ambystoma tigrinum* and what we know about the genetics and developmental biology of *Ambystoma mexicanum*. But even though host and virus genome sequences can yield insights into the intimate biology of ATV in tiger salamanders, a pathogen's effect on its host population is also a function of the environment, including host population dynamics.

Host-pathogen Population Dynamics

Not all infectious diseases are density-dependent, and not all amphibian H-P systems fit a susceptible-infectious-resistant (SIR) paradigm, but this basic theory affords a useful background for framing the problem. The extinctions of several amphibian species are thought to be a result of infectious disease, but "most simple epidemiological models indicate that there is a host-threshold density below which a pathogen cannot invade a host population, suggesting that rare or depleted populations should be less subject to invasion…" (Lafferty & Gerber, 2002). When coupled with the density-dependent nature of transmission, basic epidemiological theory suggests that pathogens are unlikely to cause extinction (Dobson & May, 1986). How can the particular details of host-pathogen systems explain this apparent contradiction? What conditions increase the risk of extinction from chytridiomycosis? Answering these questions will explain how pathogens could be responsible for the decline and extinction of some amphibian populations.

The first thing to note is that disease may not act alone in eliminating a species. Pathogens could reduce population density to levels where stochastic or deterministic factors cause extinction. Additionally, a host and pathogen are usually part of a community where one or more alternate hosts could act as reservoirs, relaxing the dependence of transmission rate on single-host density. Reservoirs are a powerful means by which one host species may suffer little disease while harboring a parasite that might drive an alternate host species to extinction (Woodroffe, 1999); conversely, multiple reservoirs could dilute a pathogen's effectiveness and reduce the chance of an epizootic (Schmidt & Ostfeld, 2001). These divergent outcomes depend on the number of subsequent infections produced by the first infected individual to appear in the population (Anderson & May, 1982). Under these circumstances we must know transmission rates within- and between-species (Dobson & Foufopoulos, 2001), making disease dynamics a property of the entire community. The complexities of understanding community disease dynamics, however, are staggering. A model system would ideally be simple enough to be tractable, yet incorporate the essential features of other disease systems. ATV in tiger salamanders appears to be just such a system.

ATV is transmitted between animals by direct contact or, at least in the laboratory, via water previously holding an infected animal; there are no vectors. Nor are there reservoir species, at least not within the regions of our studies. ATV infects other salamander species, but none of the several frog species we have experimentally challenged maintain virus replication (Jancovich *et al.*, 2001). Fish may permit replication (Schock, unpublished data), but fish and tiger salamanders do not coexist for long. There is no evidence of long-term persistence of ATV in the environment outside of salamanders: within weeks the virus becomes undetectable in pond water and dry mud. Thus, within our study areas we have a single host-parasite system.

Larval and metamorphosed animals infected with ATV usually die within weeks of infection (Jancovich *et al.*, 1997; 2001). ATV causes annual epidemics that can decimate a salamander population (Collins *et al.*, 1988). These epidemics are observed within the aquatic, primarily larval segment of the population. After the larvae metamorphose and disperse, host densities are quite low for many months. Thus we face, in a slightly different guise, the classic question of how a virulent disease that apparently drives its host population to very low densities is maintained. We suspected that in hosts with a complex life cycle, such as amphibians, one life history stage could be an intraspecific reservoir for another stage, which led to the hypothesis that relative to the

evolution of viral virulence larvae may be the "host" and the metamorphosed adults the "reservoir" (Brunner *et al*., 2003). As far as the parasite is concerned, each amphibian life stage is effectively a different organism: larvae are abundant in dense populations that are short-lived and spatially restricted, while metamorphosed animals are much less dense and scattered in diffuse populations that are long-lived and vagile.

To study the salamander-virus interaction, we first used an infection experiment to determine the relative susceptibility of the two phenotypes (Brunner *et al.*, 2003). Sibling larvae and metamorphosed animals (to minimize genetic differences in susceptibility) were exposed to a viral dose sufficient to kill half of the sample. The results showed that metamorphosed animals, our putative "reservoir," were quite sensitive to the virus. More importantly, of those that survived the infection about 30% harboured chronic, sublethal infections for over five months. We then tested if these sublethal infections were the means of viral persistence. Surviving, sublethally infected animals were housed with naïve animals to determine if their infections were transmissible – they were in four of ten cases.

Salamander-to-salamander transmission was a means of viral persistence at least in the laboratory. In the field drift fences captured young-of-the-year as they dispersed from a study pond after an epidemic. Over a nine-day sampling period the prevalence of infection varied from 46-100% (n=77 animals); 25% displayed signs of infection and 78% tested positive for virus demonstrating that asymptomatic animals may still be infectious. As in the laboratory, metamorphosed animals were highly susceptible to ATV infection, but were metamorphosed salamanders returning to the pond sublethally infected? In spring 2002 the same study site was surrounded completely with a drift fence to capture breeding, metamorphosed animals before they entered the pond. Two of 30 animals collected were infected. Later in summer 2002 there was an epidemic presumably initiated by sublethally infected animals that returned to the pond.

Conditions for the virus appear to be good in summer, but bad in winter. Little transmission likely occurs in winter when ATV persists in chronic, but transmissible infections of metamorphosed adults and juveniles. This mode of persistence is different from the "classic" definition of a reservoir since both larval and metamorphosed life history stages are required for ATV's long term persistence: larvae amplify viral prevalence, and metamorphosed animals maintain the virus between epidemics. More generally, the result raises the possibility that intraspecific reservoirs may explain the persistence of parasites in, and the declines of small, isolated amphibian populations.

DISCUSSION

An amphibian iridovirus, *Ambystoma tigrinum* virus, is completely sequenced making possible a functional genomic analysis of pathogenesis in the tiger salamander – ATV system. Coupling the ATV analysis with the impressive (for wildlife) knowledge of genetics and development available for ambystomatid salamanders will lead to a much fuller understanding of the H-P interactions that lead to infection, virulence, and transmission. Since tiger salamanders are widespread, abundant, and easily maintained in the laboratory, experiments manipulating aspects of host and parasite are possible.

Studies of ATV in tiger salamanders in the wild have uncovered some novel insights into infectious disease in amphibian populations. In theory, virulent parasites need a reservoir host to persist in infected populations that are small, which raises the question: in hosts with complex life cycles like amphibians, can one life history stage be an intraspecific reservoir for another stage? Larval and adult life stages of amphibians can act as two different "organisms" when it comes to the evolution of a pathogen's virulence. High larval densities in ephemeral sites and low densities of metamorphosed adults and juveniles suggest virulent parasite persistence is unlikely in either stage alone, but transmission between stages could maintain virulent parasites in populations. We hypothesized that larval epidemics amplify virus prevalence and sublethally infected metamorphosed animals (re)introduce virus into uninfected larval populations. Our evidence supports this hypothesis.

Ongoing studies using the molecular tools available from a fully sequenced parasite and a host with a well-studied genome and phylogeny are beginning to tease apart the various hypotheses relating to the ecology and evolution of an infectious disease in amphibians. In the future we may be able to pinpoint the gene or genes that have allowed ATV to spread in tiger salamander populations, and not into populations of frogs and other vertebrates.

There is substantial overlap between the three factors that determine H-P dynamics (Fig. 1)**.** Except for some examples involving human diseases (e.g. cholera, malaria) and some economically important agricultural models, few H-P interactions have been dissected from molecular biology to population dynamics, and even fewer in non-game wildlife. We propose that the tiger salamander – ATV system holds this potential and can be an important model for understanding how emerging infectious diseases contribute to amphibian declines, a key example of the general loss of biodiversity.

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