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Ecology, 85(2), 2004, pp. 560–566

INTRASPECIFIC RESERVOIRS: COMPLEX LIFE HISTORY AND THE PERSISTENCE OF A LETHAL RANAVIRUS

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Abstract. Virulent parasites cannot persist in small host populations unless the parasite also has a reservoir host. We hypothesize that, in hosts with complex life histories, one stage may act as an *intra*specific reservoir for another. In amphibians, for example, larvae often occur at high densities, but these densities are ephemeral and fixed in space, whereas metamorphs are long-lived and vagile but may be very sparse. Parasite persistence is unlikely in either stage alone, but transmission between stages could maintain virulent parasites in seasonally fluctuating amphibian populations.

We examined this hypothesis with a lethal ranavirus, *Ambystoma tigrinum* virus (ATV), that causes recurrent epidemics in larval tiger salamander populations, but which has no reservoir host and degrades quickly in the environment. Although exposure to ATV is generally lethal, larvae and metamorphs maintained sublethal, transmissible infections for >5 mo. Field data corroborate the persistence of ATV between epidemics in sublethally infected metamorphs. Three-quarters of dispersing metamorphs during one epidemic were infected, and apparently healthy metamorphs returning to breed harbored ATV infections. Our results suggest that larval epidemics amplify virus prevalence and sublethally infected metamorphs (re)introduce the virus into uninfected larval populations. Intraspecific reservoirs may explain the persistence of parasites in and declines of small, isolated amphibian populations.

Key words: Ambystoma tigrinum; *amphibian decline; infectious disease; intraspecific reservoir; parasite persistence; ranavirus; tiger salamander.*

INTRODUCTION

For most infectious diseases, transmission is a density-dependent process. Below a critical host population size or density, the contact rate between infected and susceptible hosts is too low to sustain a chain of transmission, and so the parasite goes extinct (Lyles and Dobson 1993). Sustained transmission is least likely for virulent parasites in slowly increasing host populations, i.e., parasites with high transmission rates and short infectious periods in populations with low rates of birth or introduction of susceptible hosts (Dye et al. 1995). Only fairly long-lived, benign parasites persist in small populations. For this reason endemic diseases are generally not considered direct threats to the small, slowly growing populations typical of endangered species (Dobson and May 1986, Lyles and Dobson 1993). Attention in conservation biology has instead focused on the introduction of novel diseases into naïve host populations (Cunningham 1996, Daszak et al. 1999, Dobson and Foufopoulos 2001) and on biological reservoirs (Cleaveland et al. 2002, Swinton et al. 2002). Reservoirs are abundant, widespread host species that can maintain virulent parasites endemically. Individuals of this reservoir species are often, but not necessarily, more resistant to disease caused by the parasite. Infection in the smaller populations of the vulnerable species then occurs when they come into contact with the reservoir. Generalist parasites with biological reservoirs are the greatest concern to conservation biologists (Cleaveland et al. 2002).

Here we present an alternative to this paradigm. In many hosts with complex life histories host densities fluctuate considerably, particularly in seasonal environments. In theory, virulent, specialist parasites should not persist long in these fluctuating populations. Yet we have observed recurrent epidemics in seasonally abundant tiger salamander populations caused by a lethal, directly transmitted ranavirus that does not remain viable in the environment as do some insect viruses (e.g., Briggs et al. 1995). We hypothesize that in hosts with complex life histories such as tiger salamanders

Manuscript received 21 June 2002; revised 9 April 2003; accepted 16 May 2003; final version received 16 June 2003. Corresponding Editor: B. Sinervo.

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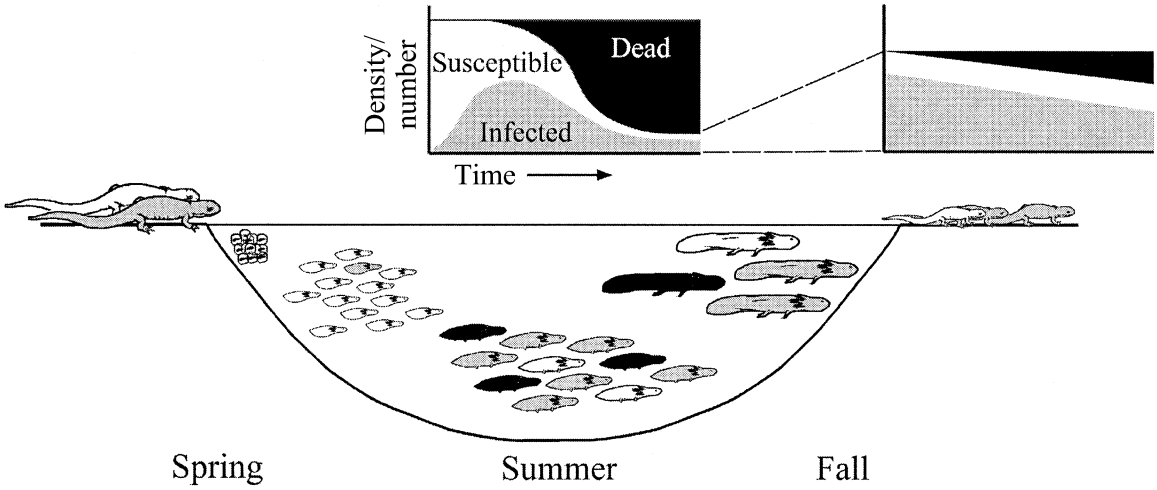

FIG. 1. A schematic of the intraspecific reservoir mechanism in tiger salamanders. Salamander larvae are dense, and transmission of *Ambystoma tigrinum* virus (ATV) is common, leading to classic epidemic dynamics. Many larvae die, but most of those surviving to metamorphosis are infected. These infected young-of-the-year metamorphs are highly susceptible to ATV, but some harbor chronic infections until they return to the pond to breed, maintaining the parasite between epidemics.

one developmental stage may introduce virulent parasites into the next, each acting as a ''reservoir'' for the other. This mode of persistence may be general to all host–parasite systems with complex host life histories, but *intra*specific reservoirs seem especially likely in amphibian hosts. Here we report laboratory studies on susceptibility and transmission complemented by field surveys that demonstrate the plausibility of this mechanism of persistence.

Host–pathogen biology

Tiger salamanders (*Ambystoma tigrinum*) breed in ponds, marshes, and earthen stock tanks in late winter or spring. Their eggs develop into quickly growing aquatic larvae that metamorphose in late summer (Collins 1981). Metamorphosed salamanders overwinter in terrestrial burrows near the ponds (Petranka 1998). In spring, mature metamorphosed salamanders return to ponds to breed, remaining there for a few days to months in the western United States (Collins 1981, Berna 1990). Hundreds of thousands of larvae may hatch, but often less than 1% survive to metamorphosis, resulting in seasonally fluctuating population densities (Collins 1981).

A ranavirus, *Ambystoma tigrinum* virus (ATV), causes recurrent die-offs in the aquatic, primarily larval segment of tiger salamander populations throughout western North America (Fig. 1; Jancovich et al. 1997, Bollinger et al. 1999, Green et al. 2002; Collins et al. 2003). These epidemics have been observed for over two decades in Arizona, usually in the late summer or early fall (Collins et al. 1988, Berna 1990, Pfennig et al. 1991). The same pond may experience epidemics several years in a row; each epidemic can kill most larvae in a year class (J. Brunner and J. Collins, *personal observations*).

ATV produces systemic infections and is generally lethal within 2–3 wk of infection (Jancovich et al. 1997, Bollinger et al. 1999). Transmission of ATV occurs via close contact with infected salamanders or immersion in water previously housing infected salamanders (Jancovich et al. 1997, Bollinger et al. 1999). ATV degrades quickly in mud and pond water (J. L. Brunner and D. M. Schock, *unpublished data*). We have no evidence of vertical transmission as virus has not been found in eggs collected from ponds with subsequent epidemics (J. L. Brunner and D. M. Schock, *unpublished data*). ATV does not have a reservoir host in our study areas. Although ATV causes infections in other salamander species, none of these are syntopic with tiger salamanders in our study areas (Jancovich et al. 2001).

Intraspecific reservoir hypothesis

Metamorphosis causes drastic changes in amphibian physiology, metabolism, immunology, etc. (Duellman and Trueb 1986, Rollins-Smith 1998), and therefore larvae and metamorphs potentially represent distinct hosts for a parasite. More importantly, larvae and metamorphs usually live in distinct habitats at very different densities (Duellman and Trueb 1986, Petranka 1998), differences that likely have strong effects on the transmission dynamics of infectious disease. Amphibian larvae are generally more numerous and occur at greater densities than metamorphosed juveniles or adults, which facilitates high transmission rates. However, because the aquatic larvae of many amphibian

species metamorphose, often less than a year after hatching, larvae are only present for part of the year. Most of an amphibian's life is usually spent in the metamorphosed form. Metamorphs are not restricted to ponds, but outside of breeding they tend to be at very low densities.

A virulent, directly transmitted parasite would have difficulty persisting in just the larval stage, because this stage is ephemeral, or just the metamorphosed segment of the population, because animals are dispersed and usually at densities that preclude frequent transmission. When these stages are combined, however, the case for persistence is qualitatively different. High larval densities would amplify the prevalence of parasite infection (in this case, the virus) while metamorphs maintain the parasite between epidemics (Fig. 1). The susceptibility of one stage compared to the other may strongly influence the magnitude, length, and spatial extent of epidemics and thus the population level effects of a parasite.

Materials and Methods

Susceptibility experiment

We exposed larval and metamorphosed tiger salamanders (*A. t. nebulosum*) from a single, 261-d-old, laboratory-bred clutch to a concentration of ATV expected to kill ~50% of the animals based on previous experiments (J. L. Brunner and D. M. Schock, *unpublished data*). Metamorphosed individuals had transformed between 4 and 63 d before exposure (32 ± 21 d, mean ± 1 SD). Salamanders were held individually in plastic shoeboxes with 2 L of aged tap water at 25°C with a 16:8 h, light:dark cycle. They were fed two mealworms twice per week, and their containers were changed weekly for the duration of the experiment. We randomly assigned 35 larvae and 24 metamorphosed salamanders to the treatment group and 25 larvae and 10 metamorphosed salamanders to the control group. Salamanders in the treatment group were then water-bath exposed to ~10^3 plaque-forming units/mL ATV, and the control group was exposed to an equivalent dose of cell culture growth medium for 1 wk. Animals were checked daily for symptoms of ATV infection (white epidermal papules, lesions, red and swollen limbs), metamorphosis, and mortality. Tail and liver samples of dead animals were frozen at −80°C until they could be screened for virus. The experiment was terminated 54 d postexposure and tail-clips from all of the surviving animals were frozen for virus screening. Tail-clips were taken from survivors once more at 120 d postexposure.

Transmission experiment

We then tested whether sublethally infected salamanders could transmit ATV to naïve salamanders. At 128 d postexposure each salamander that survived water-bath exposure was haphazardly paired with a control group salamander. All combinations of larvae and metamorphs were represented. Two pairs of control salamanders housed together served as controls for this experiment. Each of the 25 test and two control pairs was held in 400 mL of aged tap water in a 1-L Ziploc box (SC Johnson, Racine, Wisconsin, USA) to maximize opportunities for transmission. Water was changed weekly, and each pair was fed six mealworms twice per week. Unique toe clips allowed identification of the previously exposed and naïve animals in each pair. When a salamander died during the experiment, its partner was euthanized with an overdose of MS-222 (tricaine methanesulfonate; Argent Chemical Laboratories, Redmond, Washington, USA). The experiment was terminated after 45 d when all remaining salamanders were euthanized. Liver samples from all animals were frozen at −80°C until screened for virus. The number of replicates in this experiment was small, but ethics dictated that we use as few salamanders as possible to demonstrate that transmission is possible.

Virus isolation

Tail-clip and liver samples were screened for virus infection using cell culture techniques. Tissue samples were thawed on ice and then homogenized with a Stomacher 80 (Seward, Thetford, Norfolk, UK) in 3 mL of Eagle's minimum essential medium (MEM; Eagle, St. Louis, Missouri, USA) with 2% fetal bovine serum (FBS), Pen-Strep-Neomycin (1:100), and a pinch of diatomaceous earth to help break up tissues. Samples were then clarified by centrifuging at 9000 × *g* (1 *g* = 9.806 65 m/s^2) for 10 min and the supernatant inoculated onto monolayers of *Epithelioma papilloma cyprini* (EPC) cells in 10% FBS-MEM and Pen-Strep-Neomycin (1 : 100) (Jancovich et al. 1997). Each sample was inoculated onto three wells of a 12-well plate for the first passage and two wells of a six-well plate for the second. Samples were considered negative if no cytopathic effects were observed by the end of the second 10-d passage.

Field surveys

From 23 August to 1 September 2000, at the end of an epidemic, we set up three 6–12 m partial drift fences around Doughnut Tank on the Kaibab Plateau in northern Arizona, USA (36°34′36″ N, 112°12′40″ W). We collected tail clips from young-of-the-year metamorphs caught in the pitfall traps and released them. In the spring of 2002, from 8 April to 22 May, we completely encircled Doughnut tank with a drift fence and collected tail clips from adult salamanders returning to the pond. All tail clips were screened for virus as above.

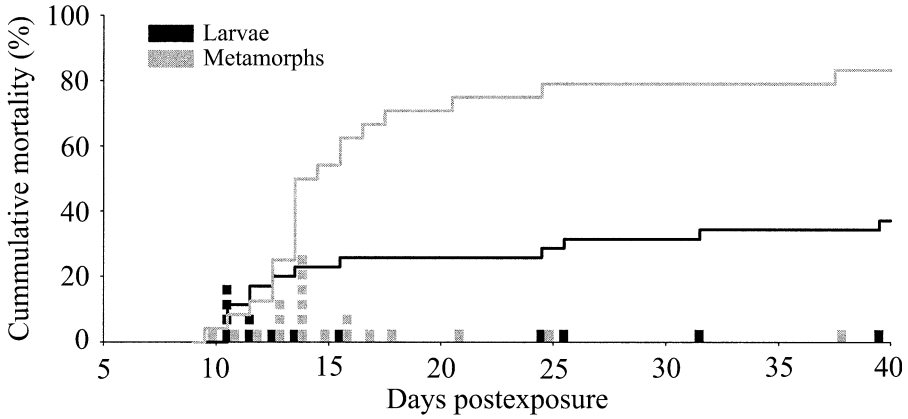

FIG. 2. Cumulative mortality of larval and metamorphosed salamanders exposed to *Ambystoma tigrinum* virus (ATV) via waterbath (lines). The boxes represent daily mortality. Experiments were conducted in a laboratory of the Arizona State University, Tempe, Arizona, USA.

RESULTS

Susceptibility experiment

No mortality occurred in the unexposed control larvae and metamorphs, and all were free of virus. Fifty-eight percent (34 of 59) of the virus-exposed animals died during the experiment, and virus infection was confirmed in all of the dead animals but one metamorph, which was therefore excluded from analyses.

Mean time to onset of symptoms and time to death did not differ between larvae and metamorphs (Fig. 2, Table 1, $t = 0.17$, $P = 0.87$ and $t = 0.69$, $P = 0.49$), but mortality was nearly twice as high in metamorphs (Table 1, $\chi^2 = 14.04$, $P < 0.001$). This could not be accounted for by differences in proportion infected as 74% of both life stages developed obvious symptoms of ATV infection such as lesions and epidermal papules (26 of 35 larvae and 17 of 23 metamorphs, $\chi^2 = 0.55$, $P = 0.46$). Rather, the differences were due to symptomatic larvae having a greater chance of losing their symptoms and surviving ($\chi^2 = 9.11$, $P < 0.01$). Half of the symptomatic larvae ''recovered'' while only one of the 17 symptomatic metamorphs recovered (Table 1). ''Recovery,'' however, was not synonymous with clearance of infection.

The mean mass of a larva at the start of the experiment was significantly greater than that of a metamorph (5.26 ± 0.88 g and 3.89 ± 0.66 g, respectively, mean ± 1 SE; $t = 6.37$, $P < 0.001$). Logistic regressions of mortality on starting mass for larvae and metamorphs separately, however, were not significant ($P = 0.30$ and $P = 0.81$, respectively), indicating that morph, rather than mass, determined the relative risk of mortality.

During the susceptibility experiment, 9 of the 35 exposed larvae transformed. There was not a significant difference in mortality between larvae that metamorphosed and those that did not (Table 1, $\chi^2 = 0.08$, $P = 0.78$), as would be expected if metamorphosis was a particularly vulnerable time. Likewise, a logistic regression of mortality on time since metamorphosis (prior to exposure) showed that recent metamorphs were

TABLE 1. Mortality, infection, and recovery of larval and metamorphosed salamanders exposed to *Ambystoma tigrinum* virus (ATV) via water bath.

Life stage	Mortality	Time to death (d)	Recovered	Sublethally infected
Larvae	13/35 (37%)	18 ± 9.6	13/26 (50%)	9/22 (41%)†
Remained larval	10/26 (38%)			
Metamorphosed	3/9 (33%)			
Metamorphs	20/23 (87%)	16.1 ± 6.2	1/17 (6%)	1/3 (33%)‡

Notes: Not all animals that died developed symptoms. Time to death data are expressed as means ± 1 SD. ''Recovered'' animals lost their apparent symptoms of ATV infection and survived. ''Sublethally infected'' animals had survived the experiment and tested positive for virus 54, 120, and/or 138–173 d postexposure. Experiments were conducted in a laboratory of the Arizona State University, Tempe, Arizona, USA.

† Seven recovered, and two were asymptomatic.

‡ One was asymptomatic.

not more vulnerable to ATV infection ($P = 0.48$). Unexpectedly, we found that after surviving ATV exposure, treated larvae were less likely to metamorphose during the experiment than their control counterparts (27% of exposed and 64% of controls, respectively; $\chi^2 = 6.34$, $P = 0.01$), although the two groups did not differ in time to metamorphosis ($t = 0.26$, $P = 0.80$).

Survivors of the susceptibility experiment were tested for virus three times: 54 d postexposure (PE), 120 d PE, and when they died or were euthanized 138–173 d PE. Virus was detected in the tissues of 10 of the 25 surviving salamanders (Table 1). Viral levels in sublethally infected individuals were frequently below detectable levels; seven of the 10 tested positive only once. Six animals tested positive 54 d PE, one was positive 120 d PE, and liver samples from six carcasses were positive 138–173 d PE. Thus, we must conclude that our estimate of the number of sublethal infections is conservative, and we cannot be certain that any ''recovered'' animals actually cleared their infections.

Transmission experiment

To test for transmission of ATV from sublethally infected animals to naïve animals, we paired each of the 25 animals that survived the susceptibility experiment with a control animal. Ten of these 25 previously exposed animals tested positive for virus and were potentially able to infect their naïve partners. Of these 10 sublethally infected animals, four individuals infected their control partners (two larvae and two metamorphs). In three of these cases the previously exposed animal appeared to relapse, developing lesions, swollen legs, and papules, after which the control animal became sick. In the other case, the control animal developed symptoms and died while the previously exposed animal remained apparently healthy.

Field surveys

At the end of an epidemic in Doughnut Tank in fall 2000, we collected 77 tail clips from dispersing young-of-the-year metamorphs. The prevalence of infection varied from 46 to 100% over the 9-d period (roughly mirroring the rising prevalence in the pond; J. L. Brunner, *unpublished data*). Overall, 25% displayed symptoms of infection, and 78% tested positive for virus, demonstrating that asymptomatic animals may still be infectious.

In spring 2002, we collected tail clips from 30 apparently healthy metamorphs returning to the pond; ATV was isolated from two. We do not know the age of these animals, but a previous drift-fencing study suggests that young-of-the-year metamorphs do not return the following year (Berna 1990), so these animals were likely not larvae in 2001. Epidemics were observed in this tank in 2000 and 2001, and there may have been others in previous years.

Discussion

Many issues need to be resolved about the specific details of this host–parasite system, but in the end the exact mechanisms are not critical to the intraspecific reservoir hypothesis. Metamorphs are more susceptible to experimental challenges with ATV, but they appear to be essential to the virus's long-term persistence in tiger salamander populations. Larvae occur in ponds for only 3–4 mo each year before metamorphosing and dispersing (Collins 1981). Our best natural history information suggest metamorphs are widely dispersed in terrestrial refugia (Collins 1981, Berna 1990), suggesting that transmission between overwintering metamorphs is rare. Laboratory experiments demonstrate that ATV does not persist in dry mud or pond water (J. L. Brunner and D. M. Schock, *unpublished data*). With no resting stage, no reservoir, and little transmission, the virus must persist in its metamorphosed host. Chronic, sublethal infections, found in 40% of the salamanders surviving laboratory exposure, lasted as long as 173 d postexposure and might have lasted longer had we not euthanized them. Additionally, these chronic infections were transmissible to naïve salamanders. Based on laboratory studies it is plausible that between epidemics ATV persists in chronically infected metamorphs.

Our field studies also support the intraspecific reservoir hypothesis. Toward the end of one epidemic on the Kaibab Plateau, 78% of the young-of-the-year were infected as they left the pond for burrows, many without apparent infections. We later found two sublethally infected adults returning to breed at this same pond, completing the virus life cycle. Most of the terrestrial life of metamorphosed salamanders is poorly known (Petranka 1998), so the origins of these two sublethally infected animals are uncertain. They may have harbored infections since leaving the pond after metamorphosis, or they may have been the end result of a short chain of transmission among metamorphs. Whatever the origins of the infection, only one infected animal need survive and return to (re)initiate a larval epidemic. Taken together, our laboratory and field studies demonstrate the plausibility of intraspecific reservoirs acting to maintain virulent parasites in host populations with complex life histories (Fig. 1).

Our proposed mechanism differs from the traditional concept of *inter*specific reservoirs. There is no host in this system that can maintain the parasite indefinitely by itself; each stage harbors the virus for the other. If not for the high prevalence in newly emerging metamorphs, a result of amplification of virus infection in larval epidemics, we would expect the virus to fade

from the metamorphosed segment of the population because most ATV infections are lethal to metamorphs. Without the recurring (re)introduction of the virus into the larvae, there would be no epidemics. Transmission between life history stages ensures ATV's persistence.

We suspect that the long-term dynamics of ATV in tiger salamander populations are strongly affected by the distinctive features of each life history stage (e.g., density, longevity, contact rates) and may vary with changes in the environment such as drought or land use changes. Drought, for example, leads to crowding among metamorphs (Alvarado 1967), which would tend to increase contact rates and transmission in burrows. A change in behavior could create a chain of transmission that ensures persistence of the virus between epidemics, but it may also amplify the effect of disease on adult hosts. Since ATV is lethal to, yet persists in, both larval and metamorphosed segments of the population, ATV may have the potential to drive tiger salamander populations to very low levels. Such declines have not been observed in tiger salamander populations, but this may simply reflect the difficulty of detecting declines in amphibian populations that vary greatly in size between years (Pechmann et al. 1991, Alford and Richards 1999).

According to basic host–parasite theory, specialized, virulent parasites cannot persist in populations below some critical host density. The intraspecific reservoir hypothesis raises the possibility that for hosts with complex life histories, virulent parasites can take advantage of transmission between the different life history stages to persist indefinitely. Although the intraspecific reservoir mechanism does not entirely circumvent the problem of critical host density, it does appear to expand the conditions under which virulent, specialized parasites can remain endemic. An intraspecific reservoir could explain how virulent parasites persist in small, isolated populations, like those of declining amphibians in pristine areas and small reserves.

ACKNOWLEDGMENTS

This work was supported in part by NSF grant #DEB 9816645 to J. P. Collins, E. W. Davidson, and A. Storfer; NSF IRCEB (Integrated Research Challenges in Environmental Biology) grant #IBN 9977063 to J. P. Collins, E. W. Davidson, and 22 collaborators; and by a Sigma Xi grant in aid of research to J. L. Brunner. Permission to construct drift fences was issued by the Kaibab National Forest and permission for field studies and collection by the Arizona Game and Fish Department. Laboratory experiments were approved under ASU animal care protocol 99–526R. Thanks to Justin Calabrese, Scott Cashins, and Alison Davis for assistance in the field; to Heidi Black for help in the laboratory; and Andrew Storfer, Matthew Parris, and Ben Bolker for helpful comments on drafts of the manuscript.

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