

## Life-History Responses to Pathogens in Tiger Salamander (*Ambystoma tigrinum*) Larvae

MATTHEW J. PARRIS,<sup>1,2</sup> ANDREW STORFER,<sup>3</sup> JAMES P. COLLINS,<sup>4</sup> AND ELIZABETH W. DAVIDSON<sup>4</sup>

<sup>1</sup>Department of Biology, University of Memphis, Memphis, Tennessee 38152, USA; E-mail: mparris@memphis.edu

<sup>3</sup>School of Biological Sciences, Washington State University, Pullman, Washington 99164-4236, USA

<sup>4</sup>School of Life Sciences, Arizona State University, Box 874501, Tempe, Arizona 85287-4501, USA

**ABSTRACT.**—We tested whether the presence of an iridovirus (*Ambystoma tigrinum* virus; ATV) could alter patterns of larval life histories in Arizona Tiger Salamanders (*Ambystoma tigrinum nebulosum*). Viral epidemics cause extreme mortality in natural populations and, thus, impose a strong selective force. We tested how exposure to ATV during larval development influences survival, growth, and frequency of cannibalism by manipulating the presence of ATV in replicated experimental tanks. ATV significantly reduced survival and larval growth. Propensity to become cannibalistic was not related to ATV exposure, suggesting that salamanders cannot facultatively respond to the presence of diseased conspecifics by reducing cannibalism. Our results demonstrate that viral pathogens may have both a direct and indirect effect on *A. tigrinum* fitness by reducing survival and growth rate.

Ecologists increasingly recognize disease as an important factor shaping host life histories (Michalakis and Hochberg, 1994; Marcogliese and Cone, 1997; Koella et al., 1998; Day, 2003), and the consequences of pathogen-induced changes in host life histories is a critical issue in the population ecology of infectious diseases (Washburn et al., 1991; Dobson and Crawley, 1994; Kohler and Wiley, 1997; Parris et al., 2004). Manipulative studies offer a powerful approach for quantifying host-pathogen dynamics and the resultant impact of disease on host life-history evolution.

The effects of pathogens on population dynamics may be especially important for amphibians in light of recent concerns about population declines (reviewed in Daszak et al., 2003). Substantial literature suggests that pathogens have played important roles in global amphibian declines (e.g., Daszak et al., 1999; Carey, 2000; Collins et al., 2003; Kiesecker et al., 2004). Disease may affect the population dynamics of several subspecies of Tiger Salamanders, including the Arizona Tiger Salamander (*Ambystoma tigrinum nebulosum*). Jancovich et al. (1997) and Brunner et al. (2004) demonstrated that a virus (*Ambystoma tigrinum* virus; ATV) might be responsible for reoccurring epizootics. ATV and the closely related Regina ranavirus (RRV) are the first pathogenic viruses described in salamanders (Iridoviridae; Anthony and Comps, 1991; Jancovich et al., 1997; Bollinger et al., 1999). Infection is spread from sick to susceptible animals through water or by direct contact (Jancovich et al., 2001). Most animals die within 2–3 weeks of first

symptoms, which include lethargy, extreme epidermal sloughing, and hemorrhaging.

Tiger Salamanders (*A. tigrinum*) are found throughout North America (Shaffer and McKnight, 1996) and are excellent models for studying effects of pathogens on life-history traits because ATV affects several subspecies (Collins et al., 2004; Storfer et al., 2004). Moreover, some subspecies of *A. tigrinum* exhibit a trophic polyphenism with two discrete larval morphs. Most hatchlings develop into a typical larval morph that feeds primarily on invertebrates, whereas some develop into cannibals (Gehlbach, 1969; Collins and Cheek, 1983). Cannibals have a broader head, large vomerine teeth, and a performance advantage relative to typicals because they can prey on conspecifics (Pedersen, 1991; Reilly et al., 1992; Loeb et al., 1994). Cannibalistic larvae also eat a wider range of other prey than typical larvae and can metamorphose earlier (an advantage in ephemeral habitats) and at larger body masses (Collins et al., 1993; Brunkow and Collins, 1996). Environmental cues influence the frequency of cannibal expression, including high intraspecific density that leads to resource competition (Maret and Collins, 1997). Disease provides a cost to cannibalism, and field studies show that there is an inverse correlation between frequency of disease and frequency of cannibalism in Tiger Salamanders throughout Arizona (Pfennig et al., 1991, 1994; Collins et al., 2003). However, it is unknown whether salamanders exhibit phenotypic plasticity, and thereby adjust cannibalism rates, in response to disease.

Our work was aimed at experimentally testing the role of a viral pathogen in an amphibian host-viral pathogen system and determining whether

<sup>2</sup> Corresponding Author.

disease can alter patterns of variation in life-history traits and affect host fitness. We also tested whether salamanders can facultatively alter the expression of the cannibalistic morph according to the infection status of conspecifics.

#### MATERIALS AND METHODS

*Source Populations and Breeding Design.*—We selected salamanders from the White Mountains of east-central Arizona (33°N, 109°W) because of field data documenting occurrences of ATV epidemics and cannibalism in the region (Pfennig et al., 1991, 1994; Brunner et al., 2004). The White Mountains consist of middle- to high-elevation subalpine grasslands and coniferous forest habitats. Salamanders use a variety of aquatic environments including small lakes, ephemeral marshes, and stock tanks (Collins, 1981). Five *A. t. nebulosum* sibships were obtained from laboratory matings between animals collected from three populations in the White Mountains. Oviposition occurred from 18 April to 1 May 2001, and larvae were combined in the following proportions determined by clutch size: Lower Cottonwood Tank (two clutches) = 64%; Wildcat Point Tank (two clutches) = 26%; South Tank (one clutch) = 10%. Clutches contained approximately 100–500 larvae each, which is within the range of clutch sizes from natural populations of *A. tigrinum* (Gehlbach, 1969; Rose and Armentrout, 1976). Sibships were combined to distribute evenly population, dam, and sire effects among all treatments. After hatching, larvae were held in plastic containers (114 liters) at an approximate density of 0.11 larvae/liter, fed brine shrimp (*Artemia* sp.) ad libitum, and reared on a 12:12 L:D h photoperiod.

*Experimental Design and Procedures.*—We raised larvae in eight polyethylene experimental tanks (1.83 m diameter) positioned in an array at the University of Memphis Edward J. Meeman Biological Field Station (Shelby County, Tennessee; 35°22'N, 90°1'W). Tanks were exposed to natural, seasonal changes in air temperature and photoperiod. Although relatively small, experimental tanks were within the size range of small ephemeral habitats in Arizona (MJP, pers. obs.). We prepared tanks in mid-May 2001 by filling them with tap water to a depth of 30 cm (750 liters), adding 1.0 kg of air-dried leaf litter collected from nearby deciduous forests, and inoculating them seven times with 500 mL aliquots of a concentrated plankton suspension collected from several nearby natural ponds. Three adult snails (Lymnaeidae) were added to each tank to graze algae on tank surfaces. These initiation procedures established complex aquatic environments with self-maintaining food webs of algae and plankton for developing salamander larvae (Parris, 1999). No water was added during the

experiment, because rainfall compensated for evaporative water loss. Securely fastened and weighted lids (fiberglass screen, 1 mm mesh) were attached to each tank and secured by attaching a tight elastic cord to provide shading and prevent colonization by predators and competitors. The relatively low water level (30 cm) minimized the probability of both pathogen and animal escape, as larvae of *A. tigrinum* and metamorphs are unable to climb the interior surfaces of experimental tanks (MJP, pers. obs.). All safety precautions were sufficient, and no further permits were necessary for this research (sensu Parris and Cornelius, 2004). We allowed tanks to condition undisturbed for 12 days before adding salamander larvae.

Treatments consisted of larvae of *A. t. nebulosum* reared in presence or absence of ATV. Larvae in all treatments were reared at an initial density of 120 larvae/tank (0.16 larvae/liter), which is within the range of natural population densities and comparable to densities used in previous experiments to induce cannibals (Collins and Cheek, 1983; Pfennig et al., 1994). The two treatment combinations were replicated either three (pathogen-free) or five (ATV-exposed) times and randomly assigned to the eight tanks. On 26 May, we haphazardly selected larvae and added them to the tanks. A sample of 10 larvae from each treatment prior to addition to the tanks indicated no significant size difference between larvae assigned to pathogen-free and ATV-exposed tanks (mean snout-vent length, SVL = 20.9 and 22.0 mm, respectively;  $t_{18} = 1.23$ ;  $P = 0.317$ ). Larvae were acclimated 10 days before applying virus treatments.

On 5 June, we placed two ATV-infected *A. t. nebulosum* larvae in a buoyant mesh cage in each pathogen treatment tank. Animals had been exposed 14 days earlier in the laboratory to water baths containing infectious viral titers ( $10^{-3}$  plaque-forming units; Jancovich et al., 1997). Infections of ATV were confirmed by inoculating a sample from each larva on a fish cell line (EPC; *Epithelioma papilloma cyprini*) using standard cell culture techniques (for details, see Jancovich et al., 1997). We passed each sample twice through cell culture and considered samples that exhibited a cytopathic effect on EPC cells as virus-positive. Infected animals were placed into cages (25 × 20 × 12 cm) constructed of untreated lumber with flexible plastic netting securely stapled on two surfaces, and cages were made buoyant by attaching tubular PVC floats at two ends. Because ATV can be transmitted through water in natural populations of *A. tigrinum* (Jancovich et al., 2001), naïve experimental larvae in our experiment could be affected by contact with water from the infected animals in the cages. Two noninfected larvae of *A. t. nebulosum*

TABLE 1. Summary of univariate analyses of variance for survival, snout-vent length (SVL) at day 75, and proportion of survivors becoming cannibalistic for uninfected and ATV-infected larvae of *Ambystoma tigrinum nebulosum* reared in experimental tanks.

Response	Source	df	MS	F	P
Survival	Infection Status	1	0.1206	6.61	0.0422
	Error	6	0.0182		
SVL	Infection Status	1	0.0099	13.88	0.0098
	Error	6	0.0007		
Cannibalism	Infection Status	1	0.0006	0.52	0.4986
	Error	6	0.0011		

were placed in control tank cages. Caged larvae did not differ significantly in SVL between pathogen-free (mean  $\pm$  1 SE =  $70.7 \pm 3.4$  mm) and viral ( $73.5 \pm 2.9$  mm) treatments ( $t_{14} = -1.01$ ;  $P = 0.670$ ). All infected, caged larvae died within 12 days of introduction into experimental tanks, at which point they were removed. On day 12, larvae from all control tank cages were also removed.

Water level in all tanks remained constant during the 75-day experimental period. There were no significant differences between minimum and maximum water temperatures at the bottom of two tanks (one at each end of the array) recorded weekly. Temperatures ranged from  $24 \pm 4$  to  $34 \pm 3^\circ\text{C}$  over the course of the experiment. We visually censused all tanks for larvae exhibiting behavioral and phenotypic symptoms of infection (e.g., lethargy, inability to submerge, pustules on body surface, rapid degeneration of gills; Jancovich et al., 1997; Parris et al., 2004) on days 12, 44, and 75. On day 75 (21 August), we removed all remaining larvae from tanks with dip nets to assess proportion of larvae becoming cannibalistic, as metamorphosed animals do not exhibit the cannibal morphology. We thoroughly disinfected all equipment throughout the experiment and tanks at the end of the experiment by adding bleach (6% sodium hypochlorite) to yield a 10% solution.

*Response Variables and Statistical Analyses.*—Proportion of individuals surviving, SVL, and the proportion of survivors becoming cannibalistic were response variables. Mean values per tank were the unit of analysis because measurements from individuals within tanks were not independent. Survival was the proportion of larvae alive on day 75. SVL at day 75 served as an index of early larval growth rate, an important component of amphibian performance (Semlitsch, 1987; Ziemba and Collins, 1999). Moreover, by day 75 sublethal ATV infections may be reflected in differential growth. Larval responses were tested for the main effect of infection status using multivariate analysis of variance for the three response variables together and then with univariate analyses of variance for each response

separately. Because density dependence and individual size variation may affect survival, growth rate and cannibal expression in larval salamanders (Polis, 1981), we tested for correlations among responses prior to conducting separate analyses of variance. Retrospective power analyses were performed for nonsignificant effects in analyses of variance. Frequencies were angularly transformed and SVL data log-transformed to ensure additivity of effects and homogeneity of error variances (Sokal and Rohlf, 1995). We included all responses in analyses of variance after normality was confirmed.

## RESULTS

There were no significant correlations among the three larval responses (survival-SVL,  $r = 0.66$ ,  $P = 0.0742$ ; survival-cannibalism,  $r = -0.01$ ,  $P = 0.9826$ ; SVL-cannibalism,  $r = 0.29$ ,  $P = 0.4878$ ), thereby justifying the use of separate analyses of variance for each larval response. Multivariate larval responses were not significantly affected by infection status (Wilk's  $\lambda = 0.237$ ,  $F_{3,4} = 4.29$ ,  $P = 0.0968$ ). ATV significantly reduced survival (Table 1, Fig. 1A) and reduced SVL at day 75 (Table 1, Fig. 1B). Infection status did not have a significant effect on the proportion of survivors becoming cannibalistic (Table 1, Fig. 1C). Statistical power to detect a significant infection status effect on cannibalism was high ( $1 - \beta = 0.6072$ ). Visual censuses of tanks containing ATV-exposed larvae indicated that  $18.8 \pm 2.7$  and  $18.2 \pm 2.1$  (mean  $\pm$  SE) larvae exhibited symptoms of infection on days 44 and 75, respectively, whereas no larvae from control tanks exhibited symptoms of infection.

## DISCUSSION

Our experiment indicates that pathogens can have a strong effect on larval life-history components. Because life history traits are closely connected to fitness, ATV is likely a selective force in populations of Tiger Salamanders. ATV reduced survival by 23%, which may lower recruitment into adult salamander populations (Semlitsch

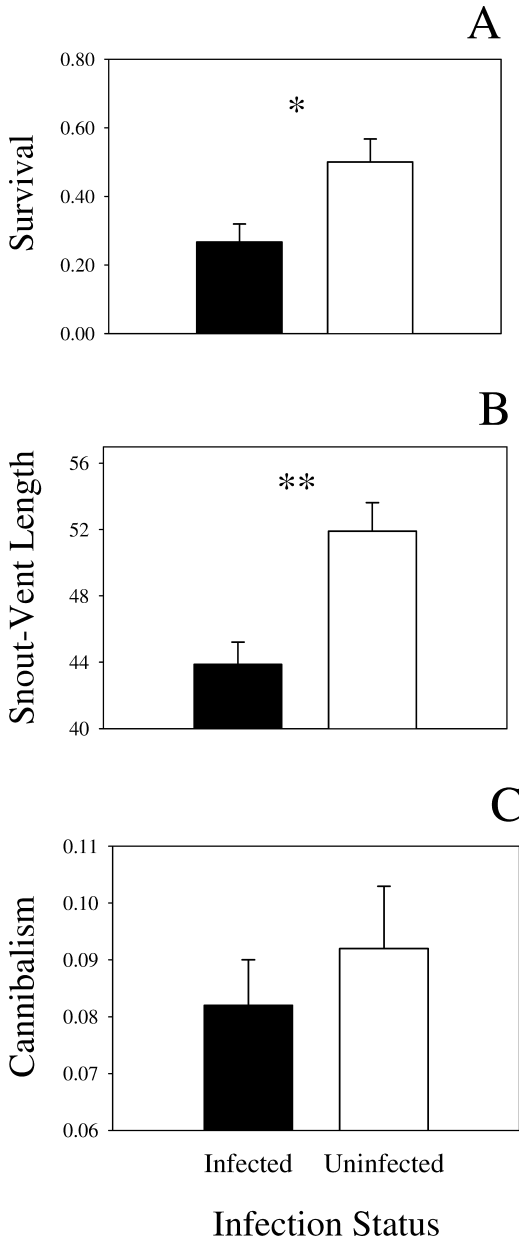


FIG. 1. (A) Proportion of individuals surviving, (B) snout-vent length (SVL) in mm at day 75, and (C) proportion of survivors becoming cannibalistic for *Ambystoma tigrinum nebulosum* larvae from the White Mountains in Arizona reared in the presence (infected) or absence (uninfected) of *Ambystoma tigrinum* virus (ATV) in experimental tanks. Values are treatment means  $\pm 1$  SE. Significant differences between infection status treatments are indicated with asterisks (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

et al., 1988). The overall potential impact of the virus on salamander populations also likely includes effects on life-history traits related to growth. In virus-exposed environments, larvae exhibited reduced body size at day 75, reflecting slower growth rates. Rapid larval growth enables pond-breeding amphibians to reach minimal size necessary to escape gape-limited predators (Wilbur, 1987; Kurzava, 1998) and initiate metamorphosis (Wilbur and Collins, 1973; Alford and Harris, 1988). When larval developmental period is prolonged, exposure to aquatic predators, competitors, and water-borne diseases is increased (Semlitsch, 1987; Semlitsch et al., 1988). In addition, failure to reach a threshold size to enable metamorphosis is a cost in ephemeral habitats (Wilbur and Collins, 1973) commonly used by Tiger Salamanders. Small size at metamorphosis also can decrease survival and reproductive success in the terrestrial environment (Semlitsch et al., 1988), potentially decreasing population growth rate (Cole, 1954).

Because cannibalism increases the risk of acquiring pathogens through feeding on infected conspecifics (Pfennig, 2000), we predicted that natural selection should minimize cannibalism in environments where pathogens are present. However, ATV did not affect the frequency of cannibals produced. The lack of plasticity for cannibalism in ATV environments may be caused by an inability to discriminate between diseased and healthy individuals. Although not addressed in our study, this mechanism is consistent with Pfennig et al. (1999), who found that cannibalistic *A. t. nebulosum* larvae did not discriminate against conspecifics infected with pathogenic bacteria in choice trials. High statistical power for the nonsignificant infection status effect on cannibalism in our experiment suggests that increased experimental replication likely would not reveal a significant effect.

Lack of plasticity in cannibalism may, thus, be a result of past selection. A high frequency of infected salamanders in a population selects against cannibalism (Polis, 1981; Pfennig et al., 1998; Pfennig, 2000) and may canalize the feeding morphology of Tiger Salamander larvae in pathogen environments. Because phenotypic plasticity often carries a cost (DeWitt et al., 1998), the lack of plasticity in cannibal expression in our experiment may be caused by historically strong disease-mediated selection against cannibalism. This is supported by field data that show an inverse relationship between disease and cannibal frequency among Tiger Salamander populations throughout Arizona (Pfennig et al., 1991, 1994; Collins et al., 2003).

Although comparable to field frequencies, cannibal expression in our experiment was low given larval densities (Maret and Collins, 1994;

Ziembra et al., 2000). Although we established initial larval densities high enough to induce high cannibal production, early mortality could lower density, potentially limiting density-dependent effects, such as cannibalism. However, the lack of a significant correlation between survival and cannibalism suggests that differential survival did not affect cannibalism. Furthermore, an analysis of covariance indicated that survival explained only a small amount (4%) of the variation in proportion of survivors becoming cannibals and is, therefore, unlikely to have had an important effect on cannibal production.

It is important to note that our experiment unambiguously isolated and tested the effect of the iridovirus ATV on larval Tiger Salamander survival and life history, whereas Pfennig et al. (1991, 1998) used field-collected water or water inoculated with pathogenic bacteria in laboratory challenge trials; the presence of virus was unknown in their experiments. Because iridoviruses have been directly implicated in salamander population die-offs (Jancovich et al., 1997; Bollinger et al., 1999), it is critical to test the effects of ATV on salamander fitness independent of other naturally occurring pathogens.

Manipulative studies in seminatural environments such as experimental tanks are of great importance for understanding the ecological complexity of host-pathogen interactions. Nevertheless, judicious use of pathogens must be evaluated in outdoor environments. By maintaining low water levels and securely fastening tank lids, we minimized probability of pathogen escape. We also thoroughly disinfected all equipment and water during and after the experiment. Our cautious approach, therefore, followed proper ethical guidelines in preventing negative ecological effects (Tiedje et al., 1989; Parris and Cornelius, 2004).

Understanding how pathogens affect life-history variation is an important aspect of developing a comprehensive theory of host-pathogen biology. Furthermore, in light of mounting evidence of amphibian population declines caused by diseases (Alford and Richards, 1999; Carey, 2000; Daszak et al., 1999, 2003), it is timely to address some of the mechanisms by which disease can affect amphibians. Our results clearly demonstrate that a viral pathogen can negatively affect life-history performance in salamanders. Future studies should incorporate additional multifactorial replicated approaches to identify the potential mechanisms by which pathogens affect phenotypic and evolutionary change in their hosts.

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