POPULATION ECOLOGY

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Dose and host characteristics influence virulence of ranavirus infections

Abstract Parasites play a prominent role in the ecology, evolution, and more recently, conservation of many organisms. For example, emerging infectious diseases, including a group of lethal ranaviruses, are associated with the declines and extinctions of amphibians around the world. An increasingly important basic and applied question is: what controls parasite virulence? We used a dose-response experiment with three laboratory-bred clutches of tiger salamander larvae (*Ambystoma tigrinum*) to test how the size of inoculum and host genetic factors influence the dynamics and outcome of ranavirus infections. We found that infection rates increased with dose and were strongly affected by clutch identity and host life history stage. Case mortality increased with dose of inoculum, but was unaffected by host characteristics. Average survival time decreased with dose and differed among clutches, but this was largely due to differences in the time to onset of symptoms. Overall, our results suggest that dose of inoculum and host characteristics (life history stage and genetic background) influence the establishment and early virus replication, and therefore the virulence of ranavirus infections.

Keywords Dose effects · Life history stage · Ranavirus · Tiger salamander · Virulence

Introduction

Given the rising number of emerging infectious diseases (Cleaveland et al. 2001; Daszak et al. 2001; Dobson and

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Foufopoulos 2001) and the suspected role of disease in the declines and extinctions of organisms (Gibbons et al. 2000; Friend et al. 2001; Daszak et al. 2003), an increasingly important question is: what controls parasite virulence? The answer is central to understanding the emergence of infectious disease and predicting the effect of parasites (broadly defined to include pathogens) on individual hosts and host populations.

Virulence is generally defined as the fitness consequences of parasite infection, but the term is usually used in the narrower sense of increased mortality rates in infected individuals (Ebert 1999). It is this restricted definition that we use in this paper. Specifically, we are interested in the average time to death of infected animals (denoted ST_{50}), which is commonly used by modelers to estimate virulence in epidemic models (virulence $\sim 1/ST_{50}$), and the probability of dying once infected (case mortality) (Day 2002b).

Although there is a great deal of theory about virulence, particularly about how virulence should evolve, the predictions from this theory depend critically on how and why infections lead to virulent outcomes (Ebert 1999; Day 2002a, b, c; Galvani 2003). Many variables can influence virulence, including host characteristics (e.g., health or life history stage; Ewald 1991; Koella and Agnew 1999; Morand and Poulin 2000), parasite characteristics (e.g., exploitation rate; Mackinnon and Read 1999; Day 2002c), host-parasite genetic interaction (i.e., Dybdahl and Storfer 2003), environmental factors (e.g., temperature or humidity; Straley and Perry 1995; Arthurs and Thomas 2001; Elliott et al. 2002), and variables related to the infection process, such as dose of inoculum (van Beek et al. 1988; Ebert et al. 2000; Timms et al. 2001).

Emerging infectious diseases, including a group of lethal ranaviruses (family *Iridoviridae*), are associated with amphibian population declines and extinctions throughout the world (Bollinger et al. 1999; Jancovich et al. 2001; Collins et al. 2003; Daszak et al. 2003). One of these ranaviruses, the *Ambystoma tigrinum* virus (ATV), and its tiger salamander host (*A. tigrinum*) are a

model system for understanding disease in amphibian populations (Collins et al. 2004).

Ambystoma tigrinum virus causes recurrent epidemics in the aquatic, primarily larval stage of tiger salamander populations throughout the Plains and Intermountain West in North America (Jancovich et al. 1997; Bollinger et al. 1999; Green et al. 2002; Collins et al. 2003). ATV infections are usually highly virulent—most infected animals succumb to ATV infection (case mortality is usually around 90%) and die within 2–3 weeks. However, like most parasites, ATV is not universally virulent. Survival times of salamanders exposed to ATV under similar conditions vary over 2 weeks and case mortality can be as low as 40% and as high as 100% (J. Brunner, unpublished data). In addition, salamanders that survive experimental ATV infections can remain asymptomatically infected, and infective to others over 5 months after initial exposure. These chronic infections seem to be important for the persistence of ATV between annual die-offs in nature (Brunner et al. 2004).

The question as to why some salamanders die quickly, others survive longer, and still others maintain chronic infections indefinitely, is key to understanding the dynamics of ATV, and perhaps other pathogens. We hypothesized that dose of inoculum explains variation in virulence. ATV is transmitted by direct contact, cannibalism, and through the water (Jancovich et al. 1997). Each route of transmission could expose salamanders to higher or lower doses of ATV, which might then lead to more or less virulent, or even qualitatively different infections (i.e., lethal vs chronic).

Dose and virulence

Virulence commonly increases with dose of parasite inoculum. Morbidity and mortality of malaria, for example, is often associated with parasite density within the host (e.g., Prybylski et al. 1999). Higher doses of viral, bacterial, and fungal pathogens increase the mortality rates and reduce the survival time of infected insects (e.g., van Beek et al. 1988, 2000; Hochberg 1991; Arthurs and Thomas 2001). Some dose-effects are purely statistical: with higher doses, the probability of a successful and potentially lethal infection increases.

Dose effects may also result from the internal dynamics of an infection. It is reasonable to assume that as parasites become more numerous in or on a host, their negative effects on the host increase. Beyond some threshold number or density, mortality may be certain (Ebert 1999; Day 2002b, c; Sabelis and Metz 2002). All else being equal, parasites with greater growth rates will reach these lethal levels sooner (e.g., Diffley et al. 1987; Chotivanich et al. 2000), as will infections starting from larger inocula (e.g., in *Plasmodium chabaudi* infections; Timms et al. 2001). This is seen more explicitly in the following equation for parasite population size assuming simple exponential growth,

$$N(t) = N(0)e^{rt}, \tag{1}$$

where $N(t)$ is the size of the parasite population at time t, $N(0)$ is the initial population size or inoculum, and r is the growth rate of the population. Solving for the time it takes the parasite population to reach a lethal level, which we will equate with the average survival time (ST_{50}), we get,

$$ST_{50} = \frac{1}{r}[\ln(N(t)) - \ln(N(0))]. \tag{2}$$

Thus, we see that average survival time is inversely related to parasite growth rate and decreases linearly with the natural log of inoculum, $\ln(N(0))$. This focus on average survival time is justified because $1/ST_{50}$ is often used as a measure of parasite virulence in SIR-type epidemic models (Day 2002b). Most SIR models, however, do not explicitly consider the influence of dose on survival time and thus ignore a potentially important aspect of host-parasite dynamics and evolutionary fitness.

Equation 2 also yields useful insights into standard dose-response experiments. If we plot survival times against the natural log of the inocula used in a dose-response experiment, we can estimate the growth rate of the parasite within (or on) a host as $r \approx -1/\text{slope}$, assuming that the parasite population grows unfettered, or at least that limitations to growth are constant. Additionally, by extrapolating this relationship to the abscissa ($ST_{50} = 0$), we can estimate the lethal parasite density or number.

When a host's immune system responds actively to infection (e.g., with cytotoxic T cells, natural killer cells, and/or antibodies), the parasite's growth rate is likely not constant and so survival time will not be linearly related to the natural log of infecting dose. Dose, however, may still affect the outcome of infections. Parasites starting from larger inocula will attain greater densities before the host can mount an adequate immune response, thereby reducing the chance of clearance and allowing the parasites to continue to grow and damage the host. Thus, case mortality may increase with dose. Conversely, infections starting with lower doses may be more easily cleared, or, if the immune response merely modulates infections, low doses may lead to chronic or sublethal infections.

With this basic theory as our guide, we conducted a dose-response experiment with three replicate clutches of tiger salamander larvae to answer two questions: (1) does virulence (time to death and case mortality) of ATV increase with dose? and (2) does low-dose exposure to ATV increase the probability of chronic infection?

Materials and methods

Design and exposure

We experimentally exposed three laboratory-bred clutches of tiger salamanders (*A. tigrinum nebulosum*)

originating from three ponds in the White Mountains of eastern Arizona, U.S.A. to a strain of ATV isolated during a die-off in the fall of 2000 on the Kaibab Plateau, Arizona (ATV-DO211). The virus was grown in *Epithelioma papilloma cyprini* cells for two passes from the original animal and titered with a plaque assay at 4.5×10^7 plaque forming units (pfu)/ml. Larvae were approximately 254 (clutch FC), 265 (clutch LC), and 400 (clutch SO) days old at the start of the experiment.

For at least 2 months before and then during the experiment, larvae were housed in individual 947-ml plastic containers (Ziploc box, S.C. Johnson, Racine, WI, USA) with ∼500–600 ml aged tap water at a room temperature of 21°C (min 19°C, max 22°C). Each salamander was fed two mealworms twice per week, and its water and plastic container was changed weekly. Larvae from each clutch were numbered and then randomly assigned to the control group, or to one of six levels of virus exposure (10^2, $10^{2.5}$, 10^3, $10^{3.5}$, 10^4 and 10^5 pfu/ml; Table 1).

The virus was serially diluted in aged tap water to create common stocks of virus-spiked water at each level. Each larva was measured (snout–vent length, SVL, in mm) and placed in an individual container with 200 ml of the appropriate virus concentration for 1 week, until its next water-change. Previous experiments indicated that the titer of ATV in the water would decline by roughly an order of magnitude over the week (J. Brunner, unpublished data). After virus exposure, we used new latex gloves when handling each animal to avoid cross-contamination. Containers and equipment were disinfected with Quat-128 (Waxie Sanitary Supply, San Diego, CA, USA).

Animals were checked in their containers daily for symptoms of infection (papules, lesions, and a stringy, sometimes bloody exudate from the cloaca), metamorphosis (gills and tail fin resorbed), and mortality. Dead animals were frozen at −80°C until later screened for infection. Surviving salamanders were euthanized with a sharp blow to the head and frozen after 39 (FC and LC) or 40 (SO) days.

Table 1 Experimental design and mortality

	Control	Dose (pfu/ml)						Total
		10^2	$10^{2.5}$	10^3	$10^{3.5}$	10^4	10^5	
FC	0/8	0/10*	1/9	2/8	5/8	8/9	9/9	25/53
LC	0/7	0/11*	6/11*	5/11	12/13	11/11	10/11	44/68
SO	0/8	2/36**	9/36**	18/35*	30/35*	32/36*	32/36	123/214
	Total	2/57	16/56	25/54	47/56	51/56	51/56	192/335

Tiger salamanders from each of three clutches were exposed to one of six doses of ATV (pfu/mL) in waterbath or to cell culture medium as a control and monitored for symptoms and/or mortality for 39 (LC and FC) and 40 (SO) days. Fractions are number dead/number in treatment. Treatment combinations with an asterisk had one (*) or two (**) dead salamanders excluded from analyses because ATV infection could not be confirmed (i.e., no symptoms were observed and PCR tests for ATV were consistently negative)

Sample preparation

Frozen carcasses were thawed on ice and ground in 10 ml of hypotonic lysis buffer (0.1 M NaCl, 0.05 M Tris–HCl, pH 8, 0.001 M EDTA) for 60 s using a Stomacher 80 (Seward Ltd., England). The location of ATV within chronically infected salamanders is uncertain, so the whole animal was ground and DNA was extracted from a 100 μl sample of this slurry. The 100 μl of slurry was incubated overnight at 37°C with 20-μl sodium dodecyl sulfate and 5-μl proteinase K. DNA was extracted with phenol:chloroform:isoamylalcohol (26:24:1) and ethanol precipitated overnight at −20°C with 0.3-M sodium acetate. DNA was concentrated by centrifugation at 14,000×g for 10 min, washed with ice-cold 70% ethanol, and resuspended in nanopure water.

Screening for virus with PCR

To determine the infection status of each sample, we amplified a ∼500 bp fragment of the 5′ end of the major capsid protein (MCP) by polymerase chain reaction (PCR) using primers #4 and #5 described by Mao et al. (1996) for FV3. PCR products were visualized by electrophoresis on 1.5% agarose gels using SYBR Green nucleic acid stain (Molecular Probes, Eugene, OR, USA) on a Dark Reader transilluminator (Clare Chemical Research, Dolores, CO, USA). Negative samples were tested twice, then re-extracted using the GeneReleaser extraction protocol (BioVentures, Murfreesboro, TN, USA) and tested for virus twice again.

Statistical analysis

Time to death and time to onset of symptoms are generally non-normally distributed, so we used the Kaplan–Meier method implemented in Proc Lifetest in SAS to generate mean survival times (ST_{50}), mean times to symptoms, and their standard errors. We then used multiple linear regression to test for dose (natural log of the inoculum) and clutch effects. In some treatment combinations, the ST_{50}'s (or mean times to symptoms) were based on very few or even single observations. We therefore weighted each mean by the proportion of animals that died (or developed symptoms) within that treatment, which had the advantage of weighting each clutch equally. Results do not vary qualitatively with different or no weighting.

We used both PCR and observation of symptoms to determine whether or not an animal became infected with ATV. These metrics were generally, but not always concordant. We therefore considered an animal as infected, if it had developed symptoms of infection and/or tested positive for ATV with PCR. The probability of infection, case mortality of these infected animals, and the probability of chronic infections (as measured by the proportion of infected survivors that were PCR positive)

were analyzed with multiple logistic regression using Proc Logistic in SAS.

Results

None of the 23 controls became infected or died. Of the 345 salamanders exposed to ATV, 202 (58.5%) died. However, ten of the 202 dead salamanders did not display symptoms and were consistently negative for ATV in multiple rounds of PCR tests. These ten salamanders of indeterminate infection status were excluded from all analyses, leaving 192 dead salamanders out of 335 overall (57.3%; Table 1). Of these 192 dead salamanders, nine displayed no symptoms but were PCR-positive for ATV, while an additional 20 salamanders developed symptoms, but survived, giving 203 symptomatic salamanders. PCR tests for ATV were generally concordant with symptoms: of the 203 symptomatic animals, 187 (92.1%) were positive by PCR, whereas only 15 (11.4%) of the 132 nonsymptomatic animals tested positive. Thus, by our inclusive measure of infection, 218 salamanders developed infections.

Infection

The best logistic regression model of infection included ln(dose) ($\chi^2 = 78.39$, df $= 2$, $P < 0.001$), clutch identity ($\chi^2 = 8.21$, df $= 2$, $P = 0.017$), whether or not a larva metamorphosed during the experiment ($\chi^2 = 7.11$, df $= 1$, $P = 0.008$), and the interaction between clutch and metamorphosis ($\chi^2 = 8.77$, df $= 2$, $P = 0.013$). This interaction was largely driven by clutch LC, where the rate of infection in the larvae was higher (44 of 54 infected, 81.5%) than in the other clutches (FC: 24 of 40, 60%; SO: 137 of 200, 68.5%), while rates of infection for metamorphosed animals were similar among clutches (FC: 3 of 13, 23.1%; LC: 3 of 14, 21.4%), though slightly elevated in clutch SO (7 of 14, 50%). In order to directly explain the influence of each factor on the probability of infection, and thus clarify the biology, we report the model with the main effects only. This approach is justifiable statistically as well since the estimated effect sizes change little between the models with and without the interaction and explained that the variation is reduced only marginally (ΔAIC $= 6.15$, $R^2 = 63.9\% \rightarrow 61.6\%$).

The odds of becoming infected increased roughly three times with every natural log increase in infecting dose (odds ratio $= 3.14$, $\chi^2 = 78.46$, $P < 0.001$; Fig. 1a), which is equivalent to a nearly 14-fold increase in odds of infection with every tenfold increase in dose. Animals from clutch FC were three times less likely to become infected than animals from clutch SO (odds ratio $= 0.33$, $\chi^2 = 6.65$, $P = 0.010$), while those from clutch LC were 1.3 times more likely to become infected (although this was only marginally significant; $\chi^2 = 3.54$, $P = 0.060$).
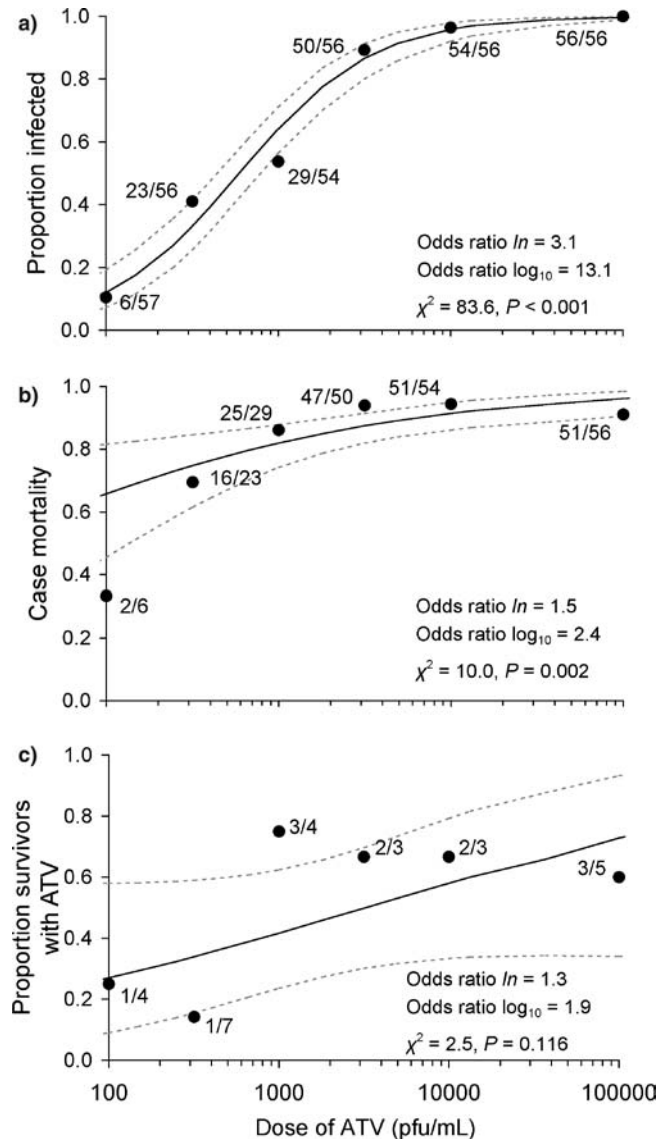
Fig. 1 Rates of infection, case mortality, and sublethal infections in tiger salamanders exposed to one of six doses of ATV. **a** Proportion of ATV-exposed salamanders that became infected (displayed symptoms of infection and/or tested positive for ATV by PCR). **b** Proportion of infected salamanders that died. **c** Proportion of the infected survivors that tested positive for ATV by PCR. *Solid lines* are probabilities from logistic regressions on ln(dose) only. *Dashed lines* are 95% CI. χ^2, and P-values are from logistic regressions

Larvae that metamorphosed during the experiment were three times less likely to become infected than those that remained larval (odds ratio $= 0.34$, $\chi^2 = 4.92$, $P = 0.027$).

Case mortality

Of the 218 infected salamanders, 192 (88.1%) died. Case mortality increased with ln(dose) (odds ratio $= 1.45$, $\chi^2 = 10.01$, df $= 1$, $P = 0.002$; Fig. 1b), but was unaffected by clutch ($\chi^2 = 2.36$, df $= 2$, $P = 0.308$) or metamorphosis ($\chi^2 = 0.001$, df $= 1$, $P = 0.981$). The odds of dying

increased ~2.4-fold for every tenfold increase in dose, from a predicted probability of 0.66 at a dose of 10^2 pfu/ml to 0.96 when exposed to 10^5 pfu/ml (Fig. 1b), although this logistic regression model explained only ~10% of the variation in mortality.

Chronic infections

Of the 26 infected salamanders that survived to the end of the experiment, 12 were PCR-positive for ATV. The probability of chronic infection did not change with ln(dose) (Fig. 1c; odds ratio = 1.33, $\chi^2 = 2.47$, $P = 0.116$), clutch ($\chi^2 = 0.37$, $P = 0.831$), or metamorphosis ($\chi^2 = 0.01$, $P = 0.910$).

Time to symptoms and death

Dose and clutch identity were statistically significant predictors of mean survival time. In the full regression model, for every tenfold increase in viral titer, animals died on average 2.87 ± 0.68 days ($X \pm 1$ SE) sooner ($\beta_{\ln(dose)} = -1.25$, $t = -4.21$, $P = 0.001$). The identity of the clutch, however, affected mean survival time to an even greater extent. Clutches FC and LC died on average 5.86 ± 1.34 days ($t = -4.38$, $P < 0.001$) and 7.28 ± 1.34 days ($t = -5.49$, $P < 0.001$) earlier, respectively, than clutch SO. Dose and clutch together explained about 77% of the variation in mean survival time.

Since we were regressing treatment means, the number of observations was reduced to 16 (five virus doses from clutches FC and LC, and six from SO), and so we did not test for interactions between dose and clutch. Separate regressions for each clutch, however, show significant or marginally significant relationships between ST_{50} and ln(dose) for the FC and SO clutches (Fig. 2a; LC: $\beta_{\ln(dose)} = -1.52$, $t = -2.41$, $P = 0.095$ and SO: $\beta_{\ln(dose)} = -1.73$, $t = -3.87$, $P = 0.018$), while the relationship for clutch LC was not significant ($\beta_{\ln(dose)} = -0.46$, $t = -1.50$, $P = 0.232$).

There was a stronger relationship between ln(dose) and the onset of symptoms (Fig. 2b). Salamanders developed symptoms on average 4.02 ± 0.52 days earlier with every tenfold increase in dose ($\beta_{\ln(dose)} = -1.75$, $t = -7.72$, $P < 0.001$). Clutch FC and LC developed symptoms 4.93 ± 1.05 and 4.65 ± 1.10 days earlier than clutch SO, respectively ($t_{FC} = -4.70$, $P < 0.001$ and $t_{LC} = -4.25$, $P = 0.001$). Dose and clutch identity together explained about 85% of the variability in average time to symptoms. The dose–time to symptoms relationship was significant for each clutch when analyzed separately (Fig. 2b; FC: $\beta_{\ln(dose)} = -1.05$, $t = -3.39$, $P = 0.027$; LC: $\beta_{\ln(dose)} = -1.48$, $t = -14.00$, $P < 0.001$; and SO: $\beta_{\ln(dose)} = -2.56$, $t = -10.05$, $P < 0.001$), suggesting that this relationship is stronger and more universal than that between ln(dose) and time to death.
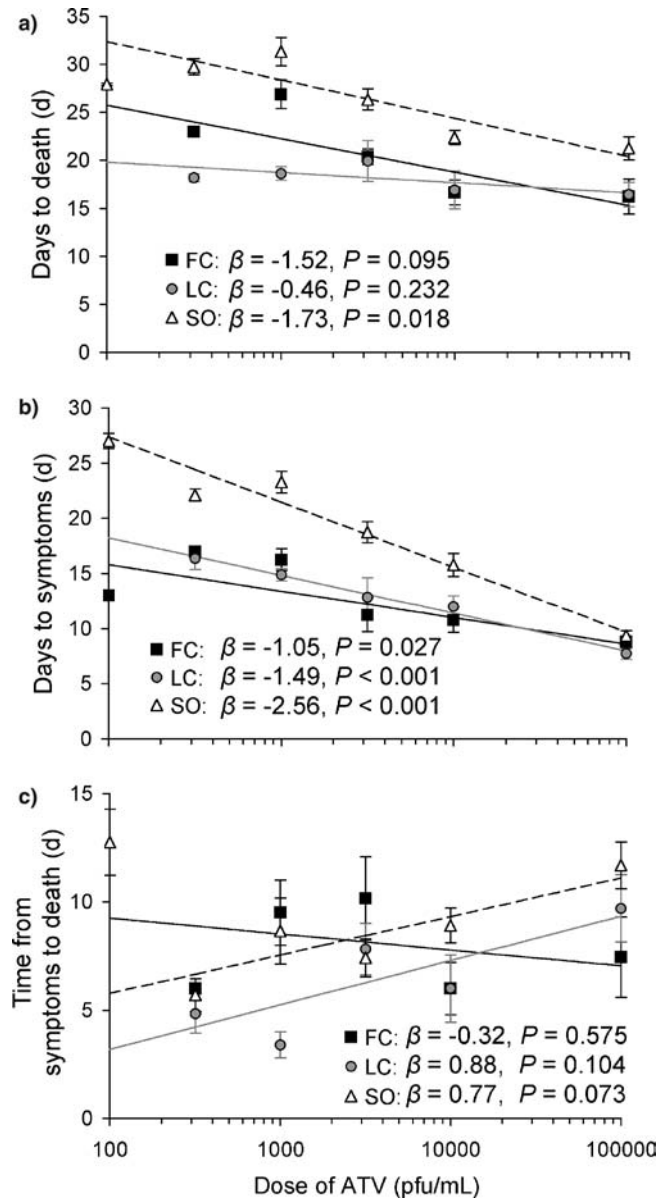
Fig. 2 Timing of events in salamanders from three clutches (FC, LC, SO) exposed to different titers of ATV. **a** Mean time to death. **b** Mean time to onset of symptoms. **c** Mean time from onset of symptoms to death. Regression lines, parameters, and P-values are from separate weighted regressions on ln(dose) for each clutch
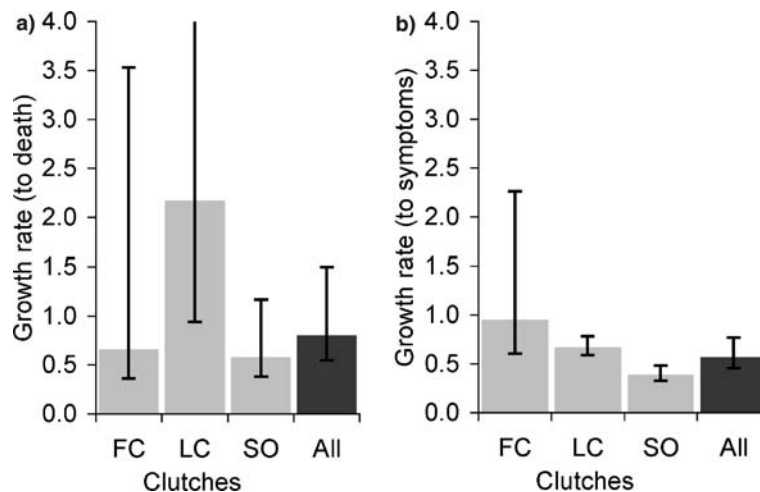
The time between onset of symptoms and mortality was unrelated to clutch ($F = 2.00$, df = 2 and 12, $P = 0.178$) and only marginally, positively related to dose (Fig. 2c; $\beta_{\ln(dose)} = 0.51$, $t = 1.98$, $P = 0.071$).

Discussion

Ambystoma tigrinum virus infections are influenced by dose of inoculum as well as host factors. Not surprisingly, the rate of ATV infection increased with dose (Fig. 1a). With increasing number of virus particles, the chances that at least one will enter and infect a

salamander increases. Clutches varied in susceptibility to infection, indicating that some host genetic factor(s) are involved in the infection process. Life history stage was also important. Salamanders that metamorphosed during the experiment (41 of 335, 12.2%) were five times less likely to become infected, perhaps due to differences between larval and metamorphosed amphibian immune systems (Cohen 1969; Rollins-Smith 1998; Carey et al. 1999). Once infected, however, case mortality was equivalent in both life history stages. In contrast, Brunner et al. (2004) found that while larval and metamorphosed salamanders both developed symptoms of infection at the same rate, metamorphs were more likely to die, although only a single clutch was used in that experiment. Future work should resolve the pattern of susceptibility in larval and metamorphosed salamanders. It would also be interesting to evaluate the relative susceptibility of neotenic tiger salamanders, which are common in some habitats where ATV epidemics are observed (Collins et al. 1988; Jancovich et al. 1997).

Case mortality among infected salamanders increased with dose; nearly 2.5-fold for every tenfold increase in dose, regardless of clutch identity or life history stage (Fig. 1b). However, dose explained only about 10% of the variability in case mortality. At least some of this lack of fit is due to case mortality leveling off at around 90% at doses of 10^3 pfu/ml and above (Fig. 1b), perhaps indicating a threshold above which dose is unimportant. Excluding the 56 observations from the highest dose improves the fit (R^2 increases from 10% to 20%, χ^2 values increase from 10.01 to 15.93), and results in a much sharper dose–case mortality relationship (odds ratio = 2.11 on natural log scale, 5.55 on a $\log_{10}$ scale); removing the 29 individuals from the lowest two doses erases the dose–case mortality relationship altogether ($\chi^2 = 0.061$, $P = 0.805$).

Surprisingly, it appears that dose did not influence the clearance of infections. Infected salamanders that survived to the end of the experiment (39 or 40 days) had roughly even odds of testing positive for ATV across all doses (Fig. 1c). With only 26 infected animals that survived to the end of the experiment, our power to detect a dose effect may be very low, or perhaps the differences among doses had not manifested themselves, and salamanders exposed to low doses were in the process of clearing the virus infections. Either way, it appears that dose influences the probability of sublethal infections indirectly by lower case mortality at lower inocula rather than via clearance of infections.

Dose of inoculum also influenced the temporal dynamics of ATV infections. In two of the three salamander clutches, increasing dose led to a smaller ST_{50}'s (Fig. 2a). A strong dose–ST_{50} relationship would be expected if ATV grows exponentially in its host to a lethal population size or density (e.g., van Beek et al. 1988; van Beek et al. 2000). In that case, the growth rate of ATV should be approximated by $r \approx -1/$slope of the regression lines in Fig. 2a (Fig. 3a). These estimates of r correspond to doubling times of ATV within its host ranging from 0.86 day to 1.2 days (excluding the estimate from clutch LC, for which the regression line was not significant).

Virulence in ATV infections of tiger salamanders, however, appears to be more complicated than simple exponential growth to a lethal threshold. Mean survival time in the LC clutch was unrelated to dose, suggesting the dose–ST_{50} relationship is not universal. More importantly, the relationship between dose and mean survival time appears to be founded on the stronger relationships between dose and time to onset of symptoms (Fig. 2b). This dose–time to symptoms relationship was significant for all three clutches, and had remarkably high R^2 values (FC: 67.8%; LC: 98%; and SO:

Fig. 3 Growth rates of ATV within tiger salamander hosts estimated as $r \approx -1/$slope of the regressions in Fig. 2. Estimates assuming that ATV grows exponentially **a** until the animal dies or **b** until symptoms are observed. *Dark gray bars* are estimates from the full regression models with ln(dose) and clutch as factors. *Error*

bars are 95% CI extrapolated from the standard errors of the slopes. Note that since the slope of clutch LC in Fig. 2(a) was nonsignificant, the 95% CI of the slope includes zero; therefore, the 95% CI of the growth rate for clutch LC in Fig. 3(a) includes infinity

95.4%). The time between symptoms and death was essentially noise that blurred the dose–ST_{50} relationship. If we assume that ATV grows exponentially until symptoms are observed, and then take the inverse of the slopes of dose–time to symptoms regression lines, we end up with estimates of ATV's growth rate in its host that are similar to those from the dose–ST_{50} regressions, only with smaller confidence intervals (CI) (Fig. 3b). These growth rates correspond to doubling times ranging from 0.73 day to 1.8 days. If these assumptions hold, ATV has a significantly lower growth rate in SO salamanders than in the other clutches.

Note that we used a conservative measure of infection: for an animal to be considered infected, it must have developed symptoms of infection and/or harbored enough ATV to be detected by PCR 39 or 40 days after exposure. Our measure incorporates both the initial establishment of ATV and the early infection dynamics. By the time we could detect an infection (e.g., an animal displayed symptoms), most of the deterministic dynamics were over. Among animals that tested positive for ATV with PCR, those that developed symptoms were 20 times as likely to die (181 of 187, 96.8%) as those that did not develop symptoms (9 of 15, 60%). Both dose and host factors seem to primarily influence the initial dynamics of ATV infections.

We hypothesize that dose and host characteristics influence ATV infections as follows. First, establishment of an ATV infection is at least partially a statistical process, such that the probability of an infection increases with increasing numbers of virions. Once inside its host, ATV must replicate. We suspect that early in an infection ATV grows exponentially, not to a lethal level, but perhaps to a level where symptoms are observed, or until some other outwardly imperceptible condition is met. This would explain why the dose–time to symptoms relationship was stronger than the dose–ST_{50} relationship. At this point, some other factor, most likely the host's immune system, counters the unrestrained growth of ATV. The timing and outcome of the infection after this point presumably depends on whether viral replication and damage to the host outstrips the host's ability to clear the virus and tolerate the destruction. A larger inoculum, however, may give the virus population a head start—case mortality increases with dose in a way that suggests a threshold above which clearance and survival is unlikely (Fig. 1b)—but the actual influence of dose on the infection occurred earlier.

Host factors also affected the early stages of infections, strongly influencing the probability of infection, but not case mortality, and the timing of symptoms and thus the timing of mortality. Clutch identity helped determine whether an animal developed symptoms (and was therefore identified as infected) and how long it took for those symptoms to arise. After a symptomatic infection had developed, however, host characteristics were unimportant. These host effects may be manifest via the host's influence on the rate of exponential growth early in the infection (e.g., via resource availability or innate immune responses), or by how and when the hosts actively respond to the infection (e.g., time to activation or lymphocyte titers). The mechanisms that generate these differences in infection and mortality rates need to be explored, but it is apparent that host factors are likely to be an important cause of variability in the virulence of ATV infections and hosts may be able to evolve increased resistance.

We suspect that this pattern of exponential growth early in an infection truncated by active host responses later on may be fairly common, particularly for viruses and bacteria that have short generation times and thus, several rounds of replication before the host can actively respond with cytotoxic T cells, natural killer cells, and/or antibodies. The simple model of exponential growth presented in the introduction (and in van Beek et al. 1988; van Beek et al. 2000) need not be rejected, but is a first approximation of the virus dynamics within a host before taking into account the active host responses.

Dose of inoculum and host factors affect the virulence of ATV infections in tiger salamanders. Virulence is often estimated as $1/ST_{50}$ in SIR models. Over the range of doses and host clutches, estimates of ATV's virulence would range from 0.032 day^{-1} to 0.062 day^{-1}. Using the more complete definition of virulence of Day (2002b) that also incorporates case mortality, virulence = CM/ST_{50}, our estimates would range from 0.005 day^{-1} to 0.062 day^{-1}. Certainly, host genetics are variable in natural populations, and we suspect that dose of inoculum varies, too, with route of exposure (e.g., direct contact vs. cannibalism or scavenging) and ecological conditions (e.g., low vs. high population densities or different temperatures). A fully formed model of ATV in tiger salamander populations would account for this variability in virulence. Overall, it appears that host ecology, life history stage, and genetic background all play roles in determining the virulence and outcome of ATV infections.

References

Arthurs S, Thomas MB (2001) Effect of dose, pre-mortem host incubation temperature and thermal behaviour on host mortality, mycosis and sporulation of *Metarhizium anisopliae* var. *acridum* in *Schistocerca gregaria*. Biocontrol Sci Technol 11:411–420

van Beek NAM, Wood HA, Hughes PR (1988) Quantitative aspects of nuclear polyhedrosis virus infections in lepidopterous larvae: the dose–survival time relationship. J Invertebr Pathol 51:58–63

van Beek N, Hughes PR, Wood HA (2000) Effects of incubation temperature on the dose–survival time relationship of *Trichoplusia ni* larvae infected with *Autographa californica* nucleopolyhedrovirus. J Invertebr Pathol 76:185–190

Bollinger TK, Mao J, Schock D, Brigham RM, Chinchar VG (1999) Pathology, isolation, and preliminary molecular characterization of a novel iridovirus from tiger salamanders in Saskatchewan. J Wildl Dis 35:413–429

Brunner JL, Schock DM, Collins JP, Davidson EW (2004) The role of an intraspecific reservoir in the persistence of a lethal ranavirus. Ecology 85:560–566

Carey C, Cohen N, Rollins-Smith L (1999) Amphibian declines: an immunological perspective. Dev Comp Immunol 23:459–472

Chotivanich K, Udomsangpetch R, Simpson JA, Newton P, Pukrittayakamee S, Looareesuwan S, White NJ (2000) Parasite multiplication potential and the severity of falciparum malaria. J Infect Dis 181:1206–1209

Cleaveland S, Laurenson MK, Taylor LH (2001) Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philos Trans R Soc Lond Ser B Biol Sci 356:991–999

Cohen N (1969) Immunogenetic and developmental aspects of tissue transplantation immunity in Urodele amphibians. In: Mizell M (ed) Biology of amphibian tumors. Springer, Berlin Heidelberg New York, pp 153–168

Collins JP, Jones TR, Berna HJ (1988) Conserving genetically-distinctive populations: the case of the Huachuca tiger salamander (*Ambystoma tigrinum stebbinsi* Lowe). In: Szaro RC, Severson KC, Patton DR (eds) Management of amphibians, reptiles, and small mammals in North America, vol GTR-RM-166. USDA Forest Service, Rocky Mountain Forest and Range Experiment Station, Fort Collins, pp 45–53

Collins JP, Brunner JL, Miera V, Parris MJ, Schock DM, Storfer S (2003) Ecology and evolution of infectious disease. In: Semlitsch R (ed) Amphibian conservation. Smithsonian Institution Press, Washington, pp 137–151

Collins JP, Brunner JL, Jancovich J, Schock DM (2004) A model host-pathogen system for studying infectious disease dynamics in amphibians: tiger salamanders (*Ambystoma tigrinum*) and *Ambystoma tigrinum* virus. Herpetol J 14:195–200

Daszak P, Cunningham AA, Hyatt AD (2001) Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Trop 78:103–116

Daszak P, Cunningham AA, Hyatt AD (2003) Infectious disease and amphibian population declines. Divers Distrib 9:141–150

Day T (2002a) The evolution of virulence in vector-borne and directly transmitted parasites. Theor Popul Biol 62:199–213

Day T (2002b) On the evolution of virulence and the relationship between various measures of mortality. Proc R Soc Lond Ser B Biol Sci 269:1317–1323

Day T (2002c) Virulence evolution via host exploitation and toxin production in spore-producing pathogens. Ecol Lett 5:471–476

Diffley P, Scott JO, Mama K, Tsen TNR (1987) The rate of proliferation among African trypanosomes is a stable trait that is directly related to virulence. Am J Trop Med Hyg 36:533–540

Dobson A, Foufopoulos J (2001) Emerging infectious pathogens of wildlife. Philos Trans R Soc Lond Ser B Biol Sci 356:1001–1012

Dybdahl MF, Storfer A (2003) Parasite local adaptation: Red Queen versus Suicide King. Trends Ecol Evol 18:523–530

Ebert D (1999) The evolution and expression of parasite virulence. In: Stearns SC (ed) Evolution in health and disease. Oxford University Press, New York, pp 161–172

Ebert D, Zschokke-Rohringer CD, Carius HJ (2000) Dose effects and density-dependent regulation of two microparasites of *Daphnia magna*. Oecologia 122:200–209

Elliott SL, Blanford S, Thomas MB (2002) Host–pathogen interactions in a varying environment: temperature, behavioural fever and fitness. Proc R Soc Lond Ser B Biol Sci 269:1599–1607

Ewald PW (1991) Waterborne transmission and the evolution of virulence among gastrointestinal bacteria. Epidemiol Infect 106:83–119

Friend M, McLean RG, Dein FJ (2001) Disease emergence in birds: challenges for the twenty-first century. Auk 118:290–303

Galvani AP (2003) Epidemiology meets evolutionary ecology. Trends Ecol Evol 18:132–139

Gibbons JW, Scott DE, Ryan TJ, Buhlmann KA, Tuberville TD, Metts BS, Greene JL, Mills T, Leiden Y, Poppy S, Winne CT (2000) The global decline of reptiles, Deja Vu amphibians. Bioscience 50:653–666

Green DE, Converse KA, Schrader AK (2002) Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001. Ann N Y Acad Sci 969:323–339

Hochberg ME (1991) Intra-host interactions between a braconid endoparasitoid, *Apanteles glomeratus*, and a baculovirus for larvae of *Pieris brassicae*. J Anim Ecol 60:51–63

Jancovich JK, Davidson EW, Morado JF, Jacobs BL, Collins JP (1997) Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. Dis Aquat Organ 31:161–167

Jancovich JK, Davidson EW, Seiler A, Jacobs BL, Collins JP (2001) Transmission of the *Ambystoma tigrinum* virus to alternate hosts. Dis Aquat Organ 46:159–163

Koella JC, Agnew P (1999) A correlated response of a parasite's virulence and life cycle to selection on its host's life history. J Evol Biol 12:70–79

Mackinnon MJ, Read AF (1999) Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*. Proc R Soc Lond Ser B Biol Sci 266:741–748

Mao J, Tham TN, Gentry GA, Aubertin A, Chinchar VG (1996) Cloning, sequence analysis, and expression of the major capsid protein of the iridovirus frog virus 3. Virology 216:431–436

Morand S, Poulin R (2000) Optimal time to patency in parasitic nematodes: host mortality matters. Ecol Lett 3:186–190

Prybylski D, Khaliq A, Fox E, Sarwari AR, Strickland GT (1999) Parasite density and malaria morbidity in the Pakistani Punjab. Am J Trop Med Hyg 61:791–801

Rollins-Smith LA (1998) Metamorphosis and the amphibian immune system. Immunol Rev 166:221–230

Sabelis MW, Metz JAJ (2002) Taking stock: relating theory to experiment. In: Dieckmann U, Metz JAJ, Sabelis MW, Sigmund K (eds) Adaptive dynamics of infectious diseases: in pursuit of virulence management. Cambridge University Press, New York, pp 379–398

Straley SC, Perry RD (1995) Environmental modulation of gene-expression and pathogenesis in *Yersinia*. Trends Microbiol 3:310–317

Timms R, Colegrave N, Chan BHK, Read AF (2001) The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. Parasitology 123:1–11