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Testing assumptions of the trade-off theory of the evolution of parasite virulence

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ABSTRACT

Background: Parasite replication is essential for transmission, but is thought to have inevitable virulent effects. The trade-off theory of parasite virulence asserts that parasites balance virulence (the increased death rate of infected hosts), which shortens the infectious period and thus reduces transmission opportunities, against transmissibility (the probability of transmission given a contact) to maximize overall transmission.

Questions: To what extent are virulence and transmissibility parasite traits? Are these traits correlated such that more virulent infections are more transmissible?

Methods: We infected tiger salamander (*Ambystoma tigrinum*) larvae with nine isolates of the *Ambystoma tigrinum* virus (ATV) and then exposed naive larvae to these infected larvae, measuring mortality rates in both to test the heritability of virulence. We then exposed five lineages of *A. tigrinum* larvae to five ATV isolates in a factorial design and measured mortality rates and virus shedding in each host–virus combination to determine the extent to which transmissibility and virulence are traits of the host and parasite, and whether they are related.

Results: Virulence is a heritable trait of virus isolates, but the variation among isolates is swamped by the much larger differences among host lineages. Transmissibility is clearly a viral trait. Within a given host lineage or across host–virus combinations there was little evidence that more virulent infections were also more transmissible. These results do not support the trade-off theory of virulence, but may reflect selection for alternative routes of ATV transmission.

Keywords: evolution of virulence, ranavirus, tiger salamander, trade-off theory, transmissibility.

INTRODUCTION

Parasite virulence, defined as the parasite-induced rate of host mortality, is often thought to be a product of parasite replication within a host. Greater replication rates in a host translate into greater virulence, but also into more transmission propagules. More virulent infections may therefore be more transmissible (i.e. more likely to be transmitted given an

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appropriate contact). There are limits to the benefits of virulence – if a parasite kills its host too soon, it will likely have few opportunities for transmission. Selection is thought to find the replication rate that balances virulence or, more precisely, the loss of transmission opportunities against transmissibility to maximize overall transmission rates, presumably at some intermediate level of virulence. This and similar trade-offs form the basis for much of the theory of the evolution of parasite virulence (Anderson and May, 1982; Bull, 1994; Ewald, 1994; Frank, 1996; Dieckmann, 2002; Galvani, 2003; Day and Proulx, 2004). However, while this theory has grown considerably and has been adapted to match more closely the realities of host–parasite systems (e.g. Day, 2001, 2002b, 2003; Day and Proulx, 2004), empirical tests of the theory are limited.

Virulence is positively correlated with transmission in several host–parasite systems (Jones *et al.*, 1996; Ebert and Mangin, 1997; Lipsitch and Moxon, 1997; Mackinnon and Read, 1999; Messenger *et al.*, 1999; Davies *et al.*, 2001; de Roode *et al.*, 2005), lending support to the trade-off hypothesis, but there are several reasons virulence may have little to do with transmission. For instance, the virulent effects of many infections [e.g. the cytokine 'storms' in response to H5N1 infections (de Jong *et al.*, 2006)] are caused by host immune responses rather than by damage done by the parasite (Graham *et al.*, 2005). Alternatively, virulent outcomes are sometimes caused when a parasite inadvertently replicates in host tissues from which it cannot be transmitted [e.g. viral and bacterial meningitis (see Levin and Bull, 1994; Lipsitch and Moxon, 1997)]. Even when parasite replication increases both virulence and transmissibility, selection may not maximize overall transmission rates. Competition between parasite genotypes within a host can instead drive the evolution of increased virulence, even at the cost of transmissibility (Antia *et al.*, 1994; Taylor *et al.*, 1998; Ebert *et al.*, 2000; de Roode *et al.*, 2005). Finally, the specific routes of transmission determine how, when or even whether a trade-off occurs. For instance, when transmission is vertical, from parent to offspring, selection tends to reduce virulence, whatever its root cause (Turner *et al.*, 1998; Messenger *et al.*, 1999). Given these complexities, it is far from certain that a trade-off between virulence and transmission is common or important to the evolution of parasite virulence (Ebert and Bull, 2003; Galvani, 2003). Empirical tests of the trade-off theory are needed.

In choosing a model system in which to test this theory, the parasite would ideally be lethal (matching the virulence term in epidemiological models from which the trade-off theory originated), killing its host by replicating to great densities, as opposed to accidentally damaging particular tissues, like the meninges, or triggering overactive host responses. It would also be directly transmitted such that host mortality represents an end to the infectious period. The tiger salamander (*Ambystoma tigrinum*)–ranavirus (*Ambystoma tigrinum* virus; ATV) system is therefore an ideal system to test evolution of virulence theory.

Ambystoma tigrinum virus is a double-stranded DNA virus that causes a systemic infection in larval and metamorphosed tiger salamanders, replicating to high titres in the gastrointestinal tract, liver, pancreas, spleen, and skin, among other tissues (Jancovich *et al.*, 1997). Infections are generally lethal within 2–3 weeks, apparently due to massive organ failure, although some individuals do apparently recover or survive with chronic infections (Brunner *et al.*, 2004). A previous study (Brunner *et al.*, 2005) demonstrated a dose-effect consistent with the premise that virulence is caused by virus replicating within the host – animals infected by larger inocula were not necessarily more likely to die, but if they were going to die, they died sooner. Transmissibility also seems to be linked to virus replication. The probability of becoming infected with ATV is clearly related to the size of the inoculum (Brunner *et al.*, 2005). Thus as more virus is shed from an infected salamander, the probability that it will infect a naive host given a potentially infectious contact should increase

(i.e. increased transmissibility). Lastly, there appears to be little genetic variation among isolates from within a region (Jancovich *et al.*, 2005), so while infection by multiple strains is possible in the laboratory (J. Brunner, unpublished data), they are unlikely in nature.

There is one way in which the ATV–tiger salamander system may not fit the trade-off theory. Trade-off theory treats virulence as a strategy or trait of the parasite, but as in many host–parasite systems the physiology of tiger salamander hosts influences the course of an infection. For instance, there was more variability in virulence among the three host lineages used in a dose–response experiment than over the three orders of magnitude of dose of inoculum (Brunner *et al.*, 2005). And, while there is no evidence of immune memory, and thus of an acquired immune response to ATV infections (J. Brunner and D. Schock, unpublished data), salamanders do sometimes recover from infections (Brunner *et al.*, 2004), indicating that interactions with the host's (presumably) innate immune system are important. How much the host and virus each contribute to the overall virulence of an infection is unclear.

We used two experimental infections to test three key assumptions of the trade-off theory of virulence evolution in the ATV–tiger salamander system: (1) virulence is predominantly a trait of the virus – hosts may resist or tolerate an infection to a greater or lesser extent, but most variation in virulence is attributable to virus isolate; (2) transmissibility, defined as the probability of transmission given an appropriate contact, is also a trait of the virus; and (3) more virulent viruses are also more transmissible. We also assessed whether total transmissibility over the duration of an infection is maximized at intermediate levels of virulence.

METHODS

Heritability study

In our first experiment, we injected groups of 10 laboratory-bred tiger salamander larvae (clutch So01, from the Mogollon Rim in Arizona) intra-peritoneally with 200 μl (1000 plaque-forming units, pfu) of one of nine ATV isolates or Frog Virus 3 (FV3; American Tissue Culture Collection #VR-567), the type of ranavirus isolated from *Rana pipiens* in the 1960s (Granoff *et al.*, 1965). The nine ATV isolates were cultured from *A. tigrinum* samples throughout western North America. Regina Ranavirus (RRV) was isolated in 1997 from Regina, Saskatchewan, Canada (Bollinger *et al.*, 1999) and COV from Mud Lake, Colorado in 2000 (Jancovich *et al.*, 2005). The Bodie virus was isolated in 1998 and Heidi7 in 1999 from ponds in the San Rafael Valley in southern Arizona. The remaining isolates were collected in 2000 from the Kaibab Plateau in northern Arizona. JMH1 was isolated from a die-off in Joe's Mud Hole, DO13 and DO81 from a die-off just a few metres away in Doughnut Tank, and SL1 and OQ1 from Snipe and Oquer Lakes, approximately 5 km from Joe's Mud Hole and Doughnut Tank. Viruses were grown in EPC (*Epithelioma papilloma cyprini*) cells to full cytopathic effects, titred by plaque assay, and frozen at −80°C until use (Jancovich *et al.*, 1997). Ten larvae injected with saline were used as controls.

Eight days into the experiment, a single naive larva was co-housed with each virus-injected larva for 2 days. The injected and co-housed larvae were separated and checked daily for signs 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 for later screening for infection using cell culture (Brunner *et al.*, 2004). Animals that survived for 69 days from the start of the experiment were euthanized with an overdose of MS-222 and then frozen at −80°C until they could be screened for infection.

Larvae were housed in individual plastic Ziploc boxes (S.C. Johnson, Racine, WI, USA) with ~900 ml aged tap water, which was changed every other day, and fed two mealworms twice per week. New latex gloves were used when handling each animal to avoid cross-contamination. Containers and equipment were washed and disinfected with a 10% bleach solution or Quat-128 (Waxie Sanitary Supply, San Diego, CA, USA).

Cross-infection experiment

The goal of this experiment was not to test for local adaptation, but to create host–parasite genetic combinations with varying levels of virulence and transmissibility. Five laboratory-bred clutches of tiger salamanders were used, two descended from animals collected from 29 Mile Lake on the Mogollon Rim in Arizona, USA (clutches 29M4 and 29M17), and three from animals collected in the White Mountains (Lower Cottonwood: clutches LC38 and LC39; Forest Canyon: clutch FC4). These lineages have been in the laboratory for several generations. Four of the five ATV isolates used in this experiment were from the Kaibab Plateau: DO17 and DO211 were both isolated from a die-off in 2000 at Donut Tank, and DO300 from a die-off at Doughnut Tank the next year. The SL1 virus is the same isolate from Snipe Lake used in the previous experiment. The Prescott virus was isolated from a die-off near Poland Junction in the Prescott Valley of Arizona in 1999, and may have been introduced into Arizona by the trade in salamanders for bait (Jancovich *et al.*, 2005). These viruses were isolated and titred by plaque assay as before, but in this case we passed each isolate ≤ 3 times from the original animal to avoid adaptation to cell culture (Ebert, 2002).

Fifty tiger salamander larvae from each of the five laboratory-bred clutches were randomly assigned to one of five virus isolates, for a sample size of ten larvae in each of the 25 host–virus combinations. Each larva was individually exposed to 200 ml of virus-spiked aged tap water (10^4 pfu $\cdot$ ml^{-1}) for one day, and then transferred to a new container with clean water and cared for as in the first experiment. Animals were checked daily for signs of infection, metamorphosis, and mortality. Dead animals were frozen at −80°C for later screening for infection with PCR (see below). Animals that survived for 29 days were euthanized with an overdose of MS-222 and individually frozen at −80°C for later screening.

To estimate the amount of virus being shed, skin scrape samples were taken from three randomly selected larvae in each of the 25 host–virus treatments ($n = 75$ larvae) every third day post-exposure for 12 days or until the animal died. Each larva was anaesthetized with MS-222 and then a sterile Teflon cell culture scraper was scraped five times along the larva's lateral surface, either left or right, alternating each sampling period. The scraper was then placed in 0.5 ml lysis buffer (below) and frozen at −80°C.

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., UK). A 200-μl sample of the resulting slurry was incubated overnight at 37°C with 20 μl sodium dodecyl sulphate and 5 μl proteinase K. DNA was then extracted using a salt-extraction protocol (Sambrook and Russell, 2001). The amount of DNA in each sample was quantified with a NanoDrop ND-1000 (NanoDrop Technologies, Delaware, USA) and diluted in sterile water to a concentration of 50 ng DNA $\cdot$ μl^{-1}. DNA was extracted from

skin scrape samples in a similar manner after the sample was vortexed and digested with 1.5 μl proteinase K.

Quantitative PCR

A quantitative real-time polymerase chain reaction (qPCR) was used to quantify viral titres in animal samples. Each sample was assayed in triplicate in 20-μl reactions containing 10 μl 2X TaqMan Universal PCR Master mix (Applied Biosystems, Inc.), 2 μl (100 ng) template DNA, 300 nmol of the forward primer rtMCP-for (5′-ACACCACCGCCCAAAAGTAC-3′), 900 nmol of the reverse primer rtMCP-rev (5′-CCGTTCATGATGCGGATAATG-3′), and 250 nmol of fluorescent probe rtMCP-probe (5′-FAM-CCTCATCGTTCTGGCCATCAACCAC-TAMRA-3′) in 384-well optical PCR plates on an ABI Prism 7900 Sequence Detection system (Applied Biosystems, Inc., California, USA). Primers and probe were designed to anneal to and amplify a 70-bp region within the major capsid protein (MCP) sequence of ranaviruses. A plasmid bearing the MCP gene from FV3 (provided courtesy of V.G. Chinchar) diluted in sterile water to concentrations of 1 to 10^7 copies per microlitre in $\log_{10}$ increments served as the standard against which unknown samples were compared. Virus titres were then measured as the number of copies of the MCP gene standardized by the amount of DNA in the real-time PCR reaction (copy number/ng DNA). Samples with a coefficient of variation greater than 15% among the three wells were re-run.

Statistical analyses

All analyses were performed in the R statistical language (R Development Core Team, 2006). Mortality rates in the two experiments were analysed using parametric survival analyses with a lognormal hazard function. Animals that survived to the end of the experiment were included in these survival analyses as right-censored observations, so they help estimate the daily hazard up to the point they were euthanized. The model syntax for the first experiment was:

survreg(Surv(I(DtoDeath), Died) ~ Virus, dist = ‘lognormal’),

where DtoDeath is the number of days from exposure to death, Died is a categorical variable indicating whether the individual died before the end of the experiment, and Virus is the virus to which the larva was exposed. We analysed mortality of the injected and co-housed larvae separately. The expected survival time of the co-housed larvae was regressed on the expected survival time of the injected larvae using least-squares regression.

For the second experiment, the fullest parametric survival model with an interaction between host clutch and virus isolate had the following syntax:

survreg(Surv(I(DtoDeath-8), Died) ~ Clutch*Virus + Meta, dist = ‘lognormal’).

Clutch and Virus are categorical variables, and Meta indicates whether or not the individual metamorphosed during the experiment. The initial 8 days during which no mortality occurred were subtracted from DtoDeath to improve the fit to a lognormal hazard.

All virus titres were transformed as $\log_{10}$(virus titre + 1). The amount of virus in carcasses was analysed using the lme4 package version 0.99875-0, maintained by D. Bates. The syntax for the fullest shedding model was:

lmer2(LogCopy ~ Virus*Day + Clutch*Day + (Day | Individual)),

where LogCopy refers to the $\log_{10}$-transformed viral titres in skin scrapes, Virus and Clutch are categorical variables, and Day is a continuous measure of the day the skin scrape was taken (3, 6, 9 or 12), which was nested within Individual. We also included a categorical variable indicating whether an animal metamorphosed during the experiment.

The virulence of individual (lethal) infections was estimated as time to death (virulence is inversely related to longevity), and the virulence in each host–virus combination as case mortality (the proportion of infected animals that died) divided by mean time to death (Day, 2002a). Transmissibility in an individual infection was measured as the mean virus titre in all of the skin scrapes taken from that individual, which essentially represents mean transmissibility. However, the rate at which infections become infectious may be a better metric of potential parasite fitness than the mean. Any factor that discounts future transmission opportunities (e.g. added sources of host mortality, epidemics that 'consume' susceptible hosts) should favour early transmission [and the virulent side-effects associated with it (Day, 2003)]. We therefore also measured transmissibility as the rate of increase in virus shedding by estimating the slope of the regression line relating virus titre to the day the skin scrape was taken. For analyses at the level of host–virus combinations, the individual means and slopes were averaged across all individuals within a host–virus combination. The relationship between virulence and transmissibility was then analysed using least-squares linear regression.

Lastly, we estimated the total amount of virus shed during an infection as a measure of total lifetime transmissibility. We simply summed the amounts of virus shed on each day of the infection, interpolating between days 3, 6, 9, and 12 (if the animal was still alive) to estimate the amount of virus shed on days we did not take measurements. Shedding was assumed to be zero at day 0, increasing linearly (on the $\log_{10}$ scale) to the amount of virus measured on day 3, and to be constant after the last measurement on day 9 or 12 until the animal died. We then regressed this estimate of the total amount of virus shed against virulence, and against virulence + virulence^2. Support for alternative versions of each model was evaluated using Akaike's Information Criterion corrected for sample size (AIC_c) and Akaike weights (Burnham and Anderson, 2002).

RESULTS

Heritability experiment

One of the ten saline-injected controls died 9 days into the experiment, apparently because of damage from the needle; virus could not be isolated from this individual, or from the other controls. None of the ten FV3-injected larvae developed signs of ranavirus infection or died before being euthanized 69 days into the experiment, and we were not able to isolate virus from these animals. All of the ATV-injected larvae presented signs of ranavirus infection and died within 20 days. The naive larvae co-housed with these ATV-injected larvae also presented signs of infection (all but five larvae) and all died within 15 days of exposure to infected larvae. Virus was recovered from all of the larvae co-housed with ATV-injected larvae, but not from any larvae paired with control or FV3-injected larvae. Time to death of the co-housed larvae increased with time to death of their injected

partners ($\beta = 0.34 \pm 0.11$, $t = 3.24$, $P = 0.002$, $R^2 = 0.097$). There was a stronger relationship between the expected times to death (from the parametric survival analyses) for each virus isolate ($\beta = 0.92 \pm 0.23$, $t = 3.93$, $P = 0.006$, $R^2 = 0.644$) (Fig. 1).

Treating the infections in the injected larvae as the first generation, the infections in the co-housed larvae as the second, and virulence (time to death) as the phenotype of an infection, these regressions are analogous to parent–offspring regressions used to estimate narrow-sense heritability, where the slope of the regression line estimates h^2 (Hedrick, 2000). The first regression using all pairs of larvae estimates the heritability of virulence in individual infections, which includes host-to-host variability as well as other sources of 'environmental' variability. The second regression, which uses average phenotypes, estimates the heritability of virulence among virus isolates or clones. Either way, it is clear that virulence is heritable. One might argue that the two routes of infection lead to very different expressions of virulence (the phenotype), for instance because of differences in dose of inoculum. Given the short and comparable times to death in both groups of larvae, it appears that both routes exposed larvae to doses of ATV above where a dose–response is observed (Brunner *et al.*, 2005). Perhaps more relevant is the relatively small amount of variability in virulence among the nine ATV isolates (Fig. 1) – from 12.3 to 15.5 days (virulence = 0.081 to 0.065 day^{-1}) when injected, and from 10.5 to 13.6 (virulence = 0.095 to 0.073 day^{-1}) when co-housed with an injected larva for 2 days.

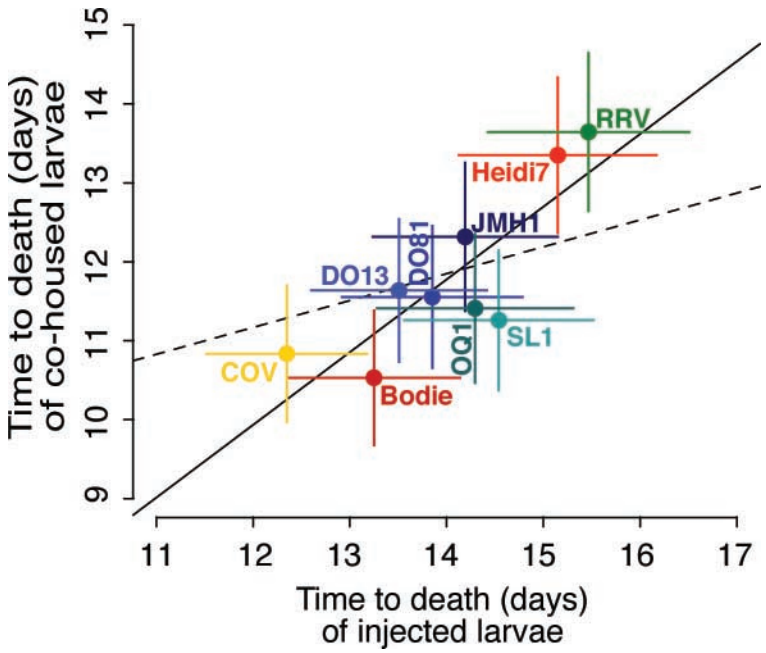

Fig. 1. Expected time to death (points) and 95% confidence interval (error bars) of larvae infected with each of nine isolates of ATV by intraperitoneal injection (abscissa) or by being co-housed with these ATV-injected larvae (ordinate). Solid line is the best-fit linear regression for these data ($\beta = 0.921 \pm 0.235$, $t = 3.93$, $P = 0.006$). Dashed line is the best-fit linear regression for individual pairs of injected and co-housed larvae ($\beta = 0.34 \pm 0.11$, $t = 3.24$, $P = 0.002$).

Cross-infection experiment

Five salamander carcasses were lost or mislabelled and thus excluded from analyses. Of the remaining 245 salamanders, 78% (191) died during the 29 days of this experiment. Mortality varied from 30 to 100% in the 25 host–virus combinations. All salamanders tested positive for ATV infection with PCR, with the exception of one that survived to the end of the experiment. It was consistently negative with standard diagnostic PCR and was just below the detection threshold in several qPCR reactions. This was the only individual that apparently recovered.

Consistent with the hypothesis that virus grows within its host to some lethal threshold concentration (Brunner *et al.*, 2005), the concentration of virus within hosts that died from ATV infection was relatively time invariant (Fig. 2), averaging about $10^{5.2 \pm 0.2}$ virus copies per nanogram of DNA in whole-body extractions. There was a slight but significant decline in virus titres in salamanders that died later ($F_{1,185} = 47.06$, $P < 0.001$). Note that this is the reverse of what would be expected if the virus continued to grow and accumulate in longer-lasting infections. This decline was driven largely by clutches FC4 and LC39, leading to a significant interaction between time to death and clutch ($F_{4,181} = 4.65$, $P = 0.001$), although clutch itself was not significant ($F_{4,185} = 1.67$, $P = 0.16$). Virus isolates did not differ in the amount of virus they produced in carcasses ($F_{4,186} = 1.52$, $P = 0.197$), and when considering time to death and virus isolate additively, only the former was significant ($F_{1,185} = 49.34$, $P < 0.001$ and $F_{4,185} = 2.19$, $P = 0.072$, respectively).

To understand the relative contributions of virus isolate and host clutch to virulence, we first used parametric survival analyses to estimate mortality rates. Virus isolate by itself was a poor predictor of time to death (compare the 'Intercept only' with the 'Virus' model in Table 1). Host clutch was a much better predictor. Nearly all of the evidentiary weight,

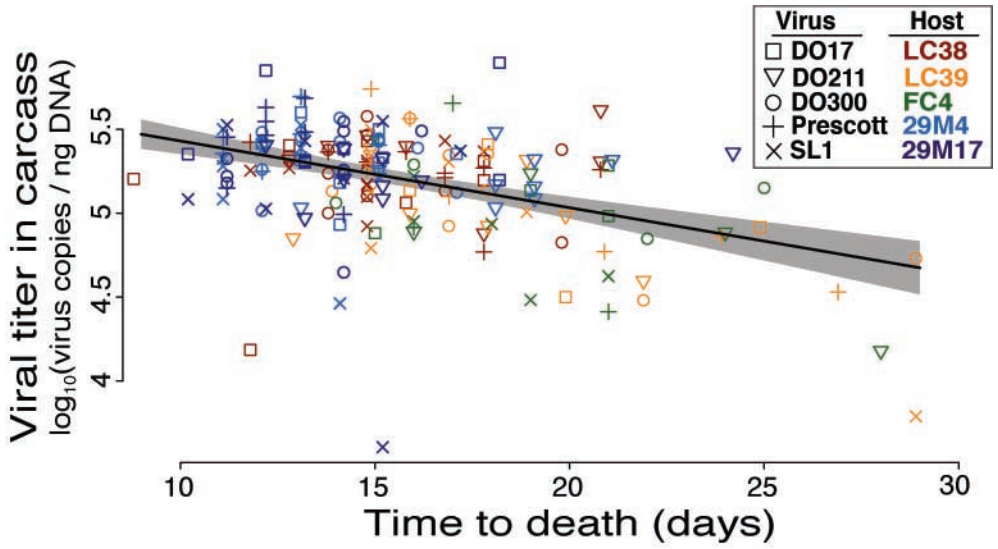

Fig. 2. Virus titre in the carcasses of tiger salamanders that died during the 29-day experiment. The shaded area is the 95% confidence interval around the solid regression line ($\beta = -0.040 \pm 0.006$, $t = -6.861$, $P < 0.001$). Neither host nor virus was significant ($F_{4,185} = 1.52$, $P = 0.191$ and $F_{4,185} = 2.19$, $P = 0.072$, respectively).

Table 1. Evidentiary support for parametric survival models

Model	p	AIC_c	Δ_i	w_i
Clutch × Virus + Metamorphosed	27	1188.6	16.4	0
Clutch × Virus	26	1195.2	23.1	0
Clutch + Virus + Metamorphosed	11	1172.1	0	0.97
Clutch + Virus	10	1181.3	9.2	0.01
Clutch + Metamorphosed	7	1179.5	7.3	0.02
Virus + Metamorphosed	7	1297.9	125.7	0
Clutch	6	1188.6	16.4	0
Virus	6	1300.8	128.6	0
Metamorphosed	3	1299.3	127.2	0
Intercept only	2	1302.4	130.3	0

Note: p is the number of parameters in the model (including the lognormal dispersion parameter, σ), AIC_c is the Akaike Information Criterion corrected for sample size, Δ_i is the difference in AIC_c from the best model, and w_i is the evidentiary weight.

however, was behind the models with the main effects of host clutch and virus isolate and the categorical variable, indicating whether the host metamorphosed during the experiment (Table 1). In this model, clutch still had a much larger effect than virus isolate: predicted median time to death varied from 14.1 to 29 days (virulence = 0.071 to 0.034 day^{-1}) among clutches while holding virus constant, and only 12.7 to 15.4 days (virulence = 0.079 to 0.065 day^{-1}) among virus isolates holding clutch constant. Salamanders that metamorphosed during the experiment lived ~1.3 days longer. There was essentially no support for the model with an interaction between clutch and virus isolate.

In separate parametric survival models for each host clutch (i.e. holding host genetic background constant), virus had a significant effect on survival time in only one of five clutches due largely to differences between virus isolates DO17 and DO211 (not shown).

We found similar results when we considered virulence [defined as case mortality divided by the mean time to death (Day, 2002a)] in each of the 25 host–virus combinations (Fig. 3). When we regressed this measure of virulence on host clutch, virus isolate or both, we once again found that virus isolate by itself was a poor predictor of virulence – the 'Virus' model had much less support than even the 'Intercept only' model (Table 2). Although the model with both clutch and virus explained ~6% more variation than the clutch only model, the clutch only model was favoured by AIC_c (Table 2), probably because the extra parameters in the fuller model were heavily penalized by small sample size ($n = 25$). (We were unable to look for an interaction between host and virus, as this model would be fully saturated.)

We measured transmissibility as the number of copies of the ATV genome (standardized by amount of DNA in the real-time PCR reaction) in skin scrapes taken from three randomly selected salamanders in each of the 25 host–virus combinations ($n = 75$ animals) on days 3, 6, 9, and, provided the animal was still alive ($n = 60$ animals), 12 days post-infection ($n = 285$ observations). Fourteen of these animals survived for the full 29 days of the experiment and were euthanized. We used repeated-measures ANOVAs with day of skin scrape nested within individual host animal to examine the influence of virus isolate and host clutch on transmissibility.

The best-supported models of virus shedding in skin scrapes included virus isolates, but not clutch (Table 3). Clutch performed worse than the model with only day of the scrape.

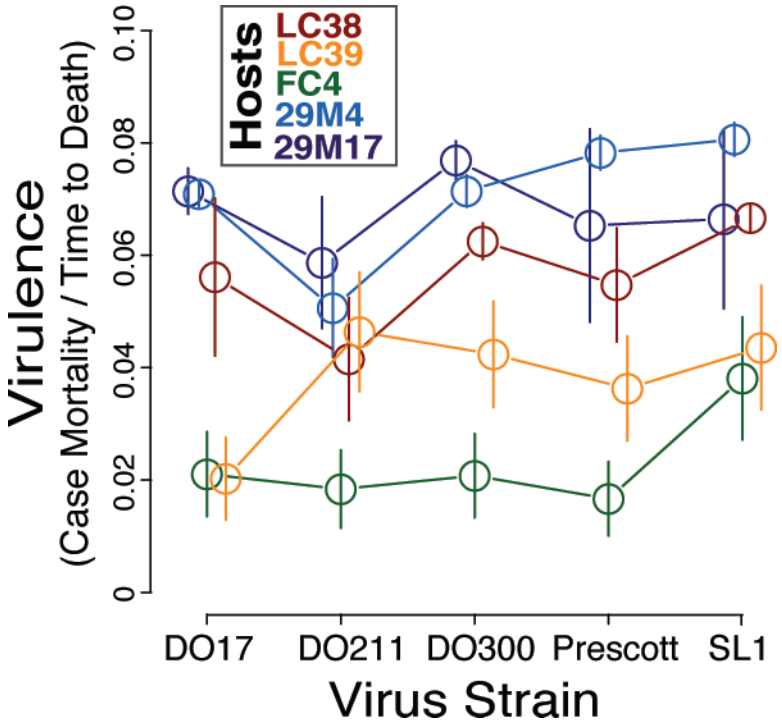

Fig. 3. Virulence (= case mortality/mean time to death) in each of the 25 host–virus combinations. Error bars are ±1 standard error as calculated using delete-one jackknife re-sampling in each combination.

Table 2. Support for regression models of virulence (= case mortality divided by mean time to death) in each of the 25 host–virus combinations

Model	p	AIC_c	Δ_i	w_i	R^2_{adj}
Clutch + Virus	10	−144.3	5.9	0.05	0.84
Clutch	6	−150.2	0	0.95	0.78
Virus	6	−109.8	40.4	0	−0.11
Intercept only	2	−119.9	30.2	0	—

Note: p is the number of parameters in the model (including the lognormal dispersion parameter, σ), AIC_c is the Akaike Information Criterion corrected for sample size, Δ_i is the difference in AIC_c from the best model, and w_i is the evidentiary weight.

Nearly all of the support fell behind the model with a virus × day-of-scrape interaction, such that amount of virus shed changed through time differently among the different virus isolates. This interaction was largely due to virus isolate DO211, which produced high titres of virus in skin scrapes on each day while the other isolates increased shedding over the 12 days ($\log_{10}$ virus copy number per nanogram DNA in skin scrapes increased by roughly 1.36 every 3 days). Excluding the DO211 isolate, the evidentiary support was strongly in favour of the additive model rather than the model with an interaction ($\Delta AIC_c = 16.8$).

Table 3. Support for repeated-measures ANOVAs of virus shedding ($\log_{10}$ virus copies per nanogram DNA in a skin scrape) through time

Model	p	AIC_c	Δ_i	w_i
Clutch × Virus + Day	29	925.8	63.7	0
Virus × Day + Clutch × Day	21	879.1	17.0	0
Clutch × Day + Metamorphosed	14	952.2	90.0	0
Virus × Day + Metamorphosed	14	866.0	3.8	0.13
Clutch × Day	13	949.1	87.0	0
Virus × Day	13	862.1	0	0.87
Clutch + Day	9	928.7	66.5	0
Virus + Day	9	908.1	45.9	0
Metamorphosed + Day	6	932.3	70.2	0
Day	5	928.3	66.3	0

Note: p is the number of parameters in the model (including the lognormal dispersion parameter, σ), AIC_c is the Akaike Information Criterion corrected for sample size, Δ_i is the difference in AIC_c from the best model, and w_i is the evidentiary weight. 'Day' is the day the skin scrape was taken, which was nested within individual animal.

In this case, the viral output differed among the four remaining virus isolates by about two orders of magnitude.

We examined the relationship between virulence and transmissibility in several ways. First, we regressed the mean amount of virus shed ($\log_{10}$ virus titre per nanogram DNA in skin scrapes averaged over all of the skin scrapes taken from an animal) against the number of days it took each of the 61 dead hosts for which we had skin scrapes to die. Mean virus shedding from these 61 animals was unrelated to time to death ('Time to Death' model, left-hand side of Table 4; $\beta_{\text{TimeToDeath}} = -0.03 \pm 0.03$, $t = -0.96$, $P = 0.344$), even when we included clutch in the model, essentially holding host genetic background constant ('Time to Death + Clutch' model; $\beta_{\text{TimeToDeath}} = -0.02 \pm 0.03$, $t = -0.64$, $P = 0.524$). Only when the virus isolate was included did time to death become a significant predictor of mean shedding ('Time to Death + Virus' model; $\beta_{\text{TimeToDeath}} = -0.05 \pm 0.02$, $t = -2.27$, $P = 0.027$), although the differences in mean virus shedding among virus isolates were much larger than those attributable to differences in time to death. Moreover, the predicted negative relationship between time to death and mean shedding was apparent in only two of five host lineages (Fig. 4, top panels). Essentially, the entire evidentiary weight was behind models in which virus isolates differed in mean shedding, with ~90% of the support behind those including time to death as well (Table 4).

If, instead, we estimate transmissibility as the rate at which virus-shedding increased (i.e. as the slope of the regression line relating virus in skin scrapes against the day the scrape was taken), transmissibility decreased with host longevity ('Time to Death' model, right side of Table 4; $\beta_{\text{TimeToDeath}} = -0.02 \pm 0.01$, $t = -2.25$, $P = 0.028$), as would be expected if higher virulence were associated with greater transmissibility. This inverse relationship between host longevity and transmissibility remained when we controlled for host genetic background ('Time to Death + Clutch' model; $\beta_{\text{TimeToDeath}} = -0.03 \pm 0.01$, $t = -2.63$, $P = 0.011$), although when the slope of the relationship was allowed to differ between clutches ('Time to Death × Clutch' model), more virulent infections were observed to be more transmissible in only three of five hosts (Fig. 4, bottom panels). Most of the evidentiary weight was behind

Table 4. Support for linear regression models of mean virus shedding estimated as the mean of the $\log_{10}$ virus titre in skin scrapes taken from an animal (left), and the rate of increase in virus shedding, estimated as the slope of the regression line relating $\log_{10}$ virus titre to the day the skin scrape was taken (right)

		Mean virus shedding			Increase in virus shedding		
Model	p	AIC_c	Δ_i	w_i	AIC_c	Δ_i	w_i
Time to Death × Virus + Clutch	15	97.9	5	0.04	−24.7	16.7	0
Time to Death + Virus + Clutch	11	95.2	2.3	0.15	−32.2	9.2	0.01
Time to Death × Virus	11	93.8	0.9	0.34	−32.8	8.6	0.01
Time to Death × Clutch	11	128.6	35.7	0	−5.4	36.0	0
Time to Death + Virus	7	92.9	0.0	0.54	−39.8	1.6	0.30
Time to Death + Clutch	7	135.1	42.2	0	−8.5	32.9	0
Virus	6	95.8	2.9	0.13	−41.4	0.0	0.68
Clutch	6	133.0	40.1	0	−3.8	37.6	0
Time to Death	3	129.9	37.0	0	−12.2	29.2	0
Intercept Only	2	128.7	35.7	0	−9.4	32.0	0

Note: p is the number of parameters in the model (including the lognormal dispersion parameter, σ), AIC_c is the Akaike Information Criterion corrected for sample size, Δ_i is the difference in AIC_c from the best model, and w_i is the evidentiary weight.

models with virus isolate included (Table 4). When we included virus isolate additively (or otherwise), the relationship between time to death and growth in virus shedding was eroded ($\beta = -0.01 \pm 0.01$, $t = -0.91$, $P = 0.366$). The increase in virus shedding appears to be related more to the specific virus isolate than to how long a particular host lived. As noted above, virus output for DO211 started very high and stayed high. Thus the slope for this virus isolate was essentially zero (inverted triangles in Fig. 4, bottom panels) while the other virus isolates all had positive slopes.

We then examined the relationship between virulence and transmission at the level of the 25 host–virus combinations. We regressed mean virus shedding (average of the individual mean virus concentrations) and the mean rate of increase in virus shedding (average of the individual slopes of virus shedding through time) against the virulence of each host–virus combination (Fig. 5, Table 5). There was no support for models relating mean virus shedding with virulence (Fig. 5a), regardless of how it was defined (left-side of Table 5; all $P \geq 0.135$). In fact, the intercept only model had equal or greater support than the virulence models. There was a somewhat stronger relationship between virulence and the rate of increase of virus production (Fig. 5b; right-side of Table 5), especially when virulence was measured as the mean time to death. This relationship was negative ($\beta = -0.03 \pm 0.01$, $t = -2.27$, $P = 0.033$, $R^2 = 0.15$), suggesting that more virulent host–virus combinations tended to ramp up virus shedding somewhat more quickly. However, this pattern was driven entirely by the near-zero slopes of DO211 viruses, particularly the FC4–DO211 host–virus combination. When this one observation was removed, the regression was no longer significant ($t = -1.32$, $P = 0.199$).

Lastly, we examined the relationship between lifetime potential transmission and virulence. We estimated lifetime *potential* transmission as the sum of virus shed during

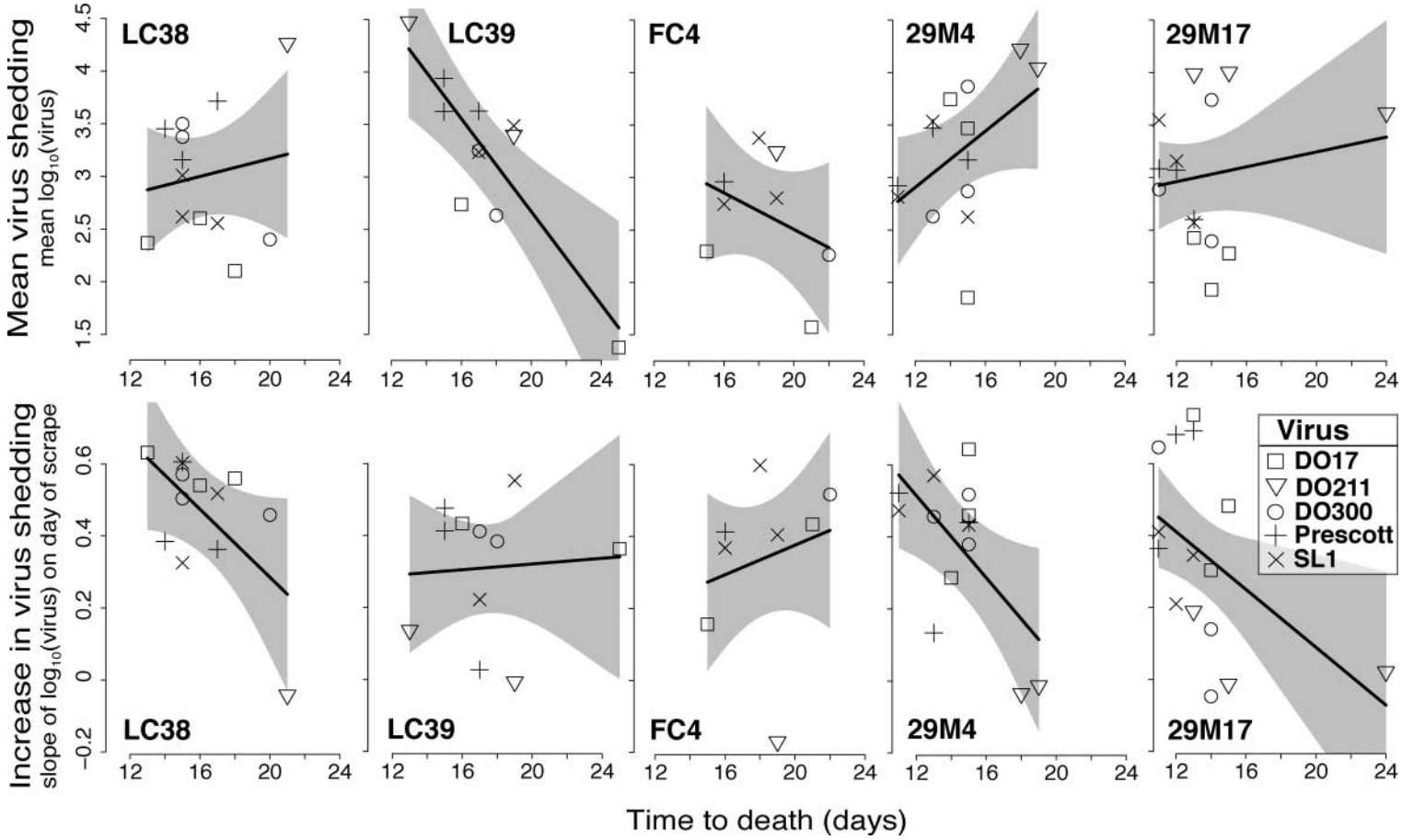

Fig. 4. Virus shedding, measured as the mean (top) or rate of increase through time (bottom) of virus titres in three to four sequential skin scrapes, plotted against time to death of the host animal for each host clutch. Lines are best-fit regression lines from the 'Time to Death × Clutch' models (Table 4). Shaded areas are confidence intervals around these regression lines.

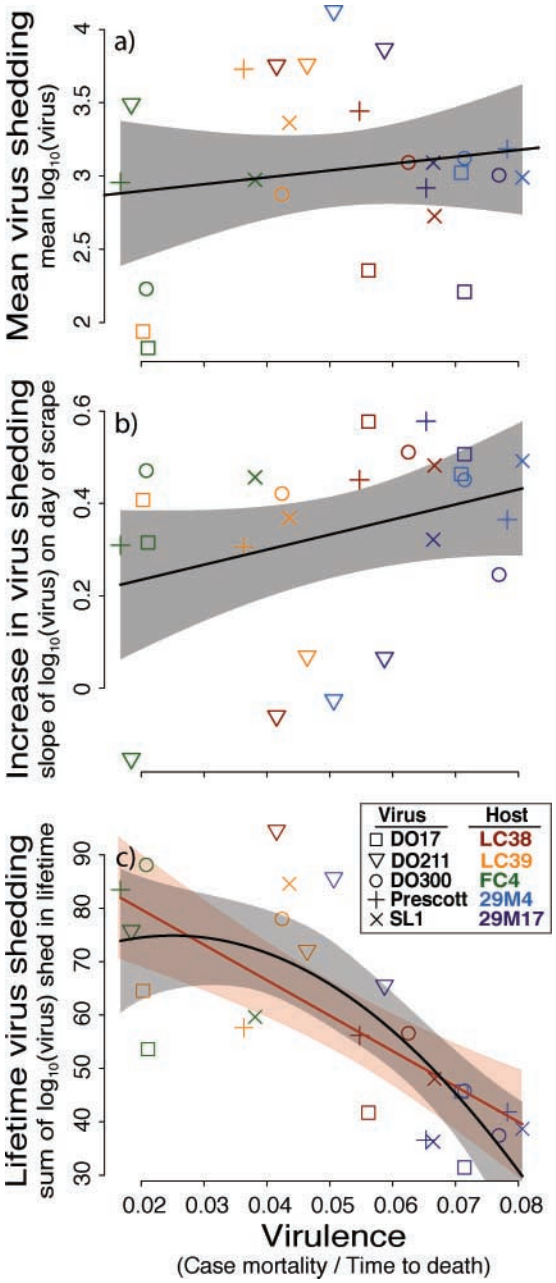

Fig. 5. The relationships between mean virulence and virus shedding (a), the rate of increase in virus shedding (b), and total lifetime virus shedding (c) in each of the 25 host–virus combinations. See text for details of estimates. The black regression lines for mean shedding (panel a) and the increase in shedding (panel b) are not significant ($t = 0.775$, $P = 0.446$ and $t = 1.67$, $P = 0.109$, respectively). The linear regression line in panel (c) is significant ($\beta = -663.0 \pm 136.5$, $t = -4.86$, $P < 0.001$), as is the second-order regression ($F_{2,22} = 15.37$, $P < 0.001$). The shaded areas are confidence intervals.

Table 5. Support for linear regression models of transmissibility, estimated as mean virus shedding (left) or as the mean rate of increase in virus shedding (right) against different estimates of virulence for each of the 25 host–virus combinations

		Mean virus shedding			Increase in virus shedding		
Model	p	AIC_c	Δ_i	w_i	AIC_c	Δ_i	w_i
Case Mortality/Time to Death	3	49.8	1.8	0.16	−6.1	2.2	0.19
Case Mortality	3	48.0	0	0.39	−4.8	3.6	0.10
Time to Death	3	50.5	2.5	0.11	−8.3	0	0.58
Intercept only	2	48.3	0.3	0.34	−5.5	2.9	0.14

Note: p is the number of parameters in the model (including the lognormal dispersion parameter, σ), AIC_c is the Akaike Information Criterion corrected for sample size, Δ_i is the difference in AIC_c from the best model, and w_i is the evidentiary weight.

an infection (see Methods) and then regressed this lifetime amount of virus shed against virulence, and against virulence + virulence^2 (Fig. 5c). There was a significant, negative relationship between virulence and lifetime virus shedding ($\beta = -663.0 \pm 136.5$, $t = -4.86$, $P < 0.001$). If we include a second-order term (virulence^2), the relationship is marginally significant ($\beta = -14604.4 \pm 7274.3$, $t = -2.01$, $P = 0.057$), but the first-order virulence term becomes non-significant ($\beta = -732.2 \pm 705.7$, $t = 1.04$, $P = 0.311$) and the evidentiary weight is equivocal ($\Delta AIC_c = 1.3$). Even in this curvilinear model, there is little evidence of a peak in potential transmission at an intermediate level of virulence (Fig. 5c).

DISCUSSION

Parasite replication is central to the trade-off theory of the evolution of parasite virulence. Replication is required to create propagules for transmission, but is thought to have inevitably virulent side-effects. In the simplest case, a parasite replicates within its host, creating more and more progeny for transmission, up to a lethal threshold number or density at which point the host dies (Brunner *et al.*, 2005). While a more virulent parasite genotype may be more transmissible (i.e. more likely to be transmitted given an appropriate contact), if it kills its host too soon, there may be few opportunities for transmission.

There may indeed be a lethal threshold for ATV in its tiger salamander hosts, as seen in the relatively constant amount of ATV in carcasses (~10^5 virus copies per nanogram of DNA; Fig. 2) as opposed to greater densities in animals that survived longer. Previous research, however, has suggested that ATV does not simply grow exponentially to this threshold, but is limited to some degree by the host (Brunner *et al.*, 2005). Given the interplay between virus replication and host responses, we cannot use final viral loads to estimate viral replication rates indirectly, as others have (e.g. de Roode *et al.*, 2007). We did, however, estimate directly the amount of virus shed and the virulence of ATV infections, which is important because the trade-off theory assumes, explicitly or otherwise, that both the number of transmission propagules produced in an infection and the virulence of that infection are principally parasite traits.

Our results clearly show that transmissibility is determined largely by the virus genotype. There was strong support for repeated-measures ANOVA models in which virus shedding

differed among isolates, while host clutch had essentially no effect (Table 3). Moreover, the rate at which virus shedding increased during an infection varied among virus isolates (Virus × Day models; Table 3), although this interaction was driven entirely by isolate DO211. This one isolate produced high shedding rates 3 days after exposure, which continued throughout the infections, while all other virus isolates, even DO17, which was isolated from the same epidemic in Doughnut Tank on the Kaibab Plateau in 2000, shed relatively little virus early on and increased shedding throughout the infections. These other isolates differed in the overall amounts of virus shed (intercepts in the Virus + Day regressions; Table 3), but followed the same general pattern.

Contrary to our expectation and results from other systems (e.g. Diffley *et al.*, 1987; Davies *et al.*, 2001; de Roode *et al.*, 2007), it does not appear that the virulence of ATV infections is principally or even largely determined by the viral genotype. While the virulence of virus isolates is highly heritable ($h^2 = 0.92$ in a common host background; Fig. 1), viruses isolated from widely disparate locations (from Arizona to Saskatchewan) varied little in mortality rates. In the cross-infection experiment, virus isolate by itself was a poor predictor of host survival time (Table 1) or virulence within a host–virus combination (Table 2; Fig. 3). Even when we held host genetic background constant, virus isolates had a significant influence on survival time in only 1 of 5 cases. Instead, most variability in virulence was due to differences among host clutches (i.e. genetic lineages). Others have found an important role of host lineage in virulence of ATV infections (e.g. Brunner *et al.*, 2005; D.M. Schock, T.K. Bollinger and J.P. Collins, submitted), but this is the first study to compare the relative importance of host and virus. Our results suggest that virus isolates have relatively little control over the outcome of infections, especially compared with the large variation in virulence among host lineages. It may be more accurate to treat survival time and case mortality as measures of the host's ability to limit or clear infections (resistance) or, given that essentially every host was infected at the end of the experiment, to limit the damage done by infections (tolerance).

The most important assumption of the trade-off theory is that, whatever the reason for variation in virulence, more virulent infections or parasite genotypes are also more transmissible. We found very little support for this assumption as well. We first examined the relationship between virulence and transmissibility among individual hosts. If virus replication was directly (or closely) related to amount of virus being shed, as well as to virulence, then virus shedding should be inversely related to time to death across all individual hosts. Even with strong host effects, we would still expect a clear, relatively consistent relationship between shedding and time to death when holding host clutch constant (i.e. the 'Time to Death + Clutch' model in Table 4 should be favoured and we should see parallel lines in Fig. 4). In fact, there was essentially no support for the 'Time to Death' or 'Time to Death + Clutch' models (Table 4). The entire support was behind models that involved virus isolate. Once virus isolate was factored out (i.e. included additively or crossed with time to death), shedding did decline with host longevity, but the effect was small compared with the differences among virus isolates. If instead we measured transmissibility as the increase in shedding throughout an infection, we found an even stronger role for virus isolates, again largely due to DO211, but only a weak relationship with virulence. Thus virulence and shedding do not seem to be strongly linked via replication *per se*.

Although there may not be a relationship between virulence and transmissibility in individual infections, it is possible that more virulent host–virus combinations are also more transmissible. This would be the case if, for instance, some virus isolates are better able to

avoid or counter host immune responses. Virulence would be a product of the interaction between host and virus, and those host–virus combinations that allowed the virus to grow unhindered would be more transmissible and more virulent. Again, we found little support for a positive relationship between virulence and transmissibility, regardless of how we measured either term (Fig. 5a, b; Table 5). The intercept only models had nearly equivalent support when transmissibility was measured as mean shedding rate. Host–virus combinations that led to death sooner appeared to ramp up virus production more quickly (larger slopes) when we measured transmissibility as the rate of increase in virus shedding. This relationship, however, was driven almost solely by the DO211 virus's apparently slow rate of increase in shedding, but this virus had already reached high levels of shedding by our first measurement on day 3, so these slopes are misleading. If we remove these isolates, or even the FC4–DO211 host–virus combination, which had the longest average survival time, this relationship disappears. Thus we find little support for a positive relationship between virulence and transmissibility at the level of host–virus combinations.

It is not surprising, then, that lifetime transmissibility of infections was not maximized at an intermediate level of virulence (Fig. 5c). Rather, at least by our metric, overall transmissibility was highest in the least virulent infections.

It is difficult to square these results with the trade-off theory of parasite virulence. Certainly, a larger sample size or more viruses and host lineages would improve the generality of our study and lead to more significant results. (Note, however, that we *did* find strong, significant relationships, just not with the expected factors.) It would have been useful to have some direct measure of virus replication within the host, as well as a more direct measure of transmission. But we feel that our results are strong enough to conclude that the trade-off model explains little if any of the variability in virulence and transmissibility in the tiger salamander–ranavirus system.

If transmission and virulence are not traded off against each other, then why is ATV virulent? Two potential explanations seem relevant. First, it is important to note that transmissibility is only one part of the transmission term in epidemiological models; the other is the contact rate (Day, 2001). *Ambystoma tigrinum* virus can be transmitted via several routes (Brunner *et al.*, 2007), but we know little about how most transmission occurs in nature (but see Greer and Collins, 2008). If lethargic, moribund salamanders have more contacts than do healthier ones [e.g. if they are unable to avoid the aggressive interactions and even cannibalism common in tiger salamander larvae (Ziemba and Collins, 1999)], selection may favour virus strains that lead to morbidity and perhaps mortality. At the extreme, when cannibalism or necrophagy are an important route of transmission, selection favours increased virulence (Day, 2003). Other ranaviruses can be transmitted when tadpoles scavenge infected carcasses (Harp and Petranka, 2006) and consumption of even small amounts of infected tissue can be an effective route of ATV transmission (Brunner *et al.*, 2007), but just how important cannibalism and necrophagy are for transmission is unclear.

A second reason virulence and virus shedding might be uncoupled is if multiple virus genotypes compete within a host. Phylogenetic analyses of ATV isolates show limited genetic variation among isolates from western North America, but local adaptation within broad geographic regions (Jancovich *et al.*, 2005), leading us to hypothesize that multiple infections, which are possible in the laboratory, would be rare in nature. Our results seem to challenge this view. If limited genetic divergence and local adaptation within regions were the norm, then the viruses from the San Rafael Valley in southern Arizona (Bodie and Heidi7 in Fig. 1) should have been phenotypically more similar to one another than to the

viruses from the Kaibab Plateau in northern Arizona (JMH1, DO13, DO81, SL1, and OQ1 in Fig. 1) in the first experiment. Instead, the two viruses from southern Arizona were among the most dissimilar in terms of virulence. Similarly, in the second experiment the DO and JMH viruses, isolated from ponds just a couple of metres apart, should have been more similar to each other than the SL1 virus from Snipe Lake (~5 km away on the Kaibab Plateau), which should have been more similar than the Prescott virus (> 250 km from Doughnut Tank), which likely originated in the salamander bait trade (Jancovich *et al.*, 2005). Instead, we found greater variability in virulence and transmissibility between the two isolates from the 2000 epidemic in Doughnut Tank (DO17 and DO211) than between viruses isolated hundreds of kilometres away. This variability could have arisen during isolation and/or serial passage in cell culture (Ebert, 2002), although all of the virus isolates experienced the same conditions and were only passed 2–3 times. If instead the differences we observed are representative of viruses in nature, and there are multiple virus strains circulating within a population with different shedding strategies and different levels of virulence, then competition among viruses for hosts could be an important factor in the evolution of virulence (e.g. Mackinnon and Read, 1999; de Roode *et al.*, 2005). As it stands, we find little evidence that there is a trade-off between transmissibility and virulence in the ATV–tiger salamander system.

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