SPATIAL VARIATION IN SELECTION AND MULTIVARIATE ESTIMATES OF LOCAL ADAPTATION IN A SALAMANDER-VIRUS SYSTEM

By

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A thesis submitted in partial fulfillment of
the requirements for the degree of

MASTER OF SCIENCE IN ZOOLOGY

WASHINGTON STATE UNIVERSITY
School of Biological Sciences

MAY 2009

To the Faculty of Washington State University:

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Andrew Storfer, and graduate committee members Mark Dybdahl and Kevin Snekvik for advice and feedback in experimental design and analysis. Jacob Kerby was an indispensable source of assistance in designing and running the experiment as well as analysis. Luke Harmon provided new insight in analysis. The Washington Disease Diagnostic Laboratory provided cell cultures and training for both virus and cell culturing. I would also like to show my gratitude to the many people who helped with lab and field work: Jason Baumsteiger, Trent Bollinger and the Bollinger Lab, Nicholas Chojnacki, Jennifer Cotter, Jonathan Eastman, Rebecca Featherkile, Denim Jochimsen, Melanie Murphy, and Steve Spear. In addition, many thanks to those who were a constant source of support, ideas, and feedback: Jason Baumsteiger, Nicholas Chojnacki, Jonathan Eastman, Denim Jochimsen, Melanie Murphy, and Steve Spear.

This research was conducted at Washington State University under all Institutional Animal Care and Use Committee guidelines, following ASAF #3331 and #3203. Funding was provided by the National Science Foundation grant DEB-0548415 under P.I. Andrew Storfer and the American Society of Ichthyologists and Herpetologists Gaige Fund (2007) to Karen M. Chojnacki (nee Benyo).

Much appreciation to the many salamanders that lost their lives to support this research. May you all have freedom in your next lives.

SPATIAL VARIATION IN SELECTION AND MULTIVARIATE ESTIMATES OF LOCAL ADAPTATION IN A SALAMANDER-VIRUS SYSTEM

Abstract

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May 2009

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It is expected that pathogens should be locally adapted to their hosts because of higher evolutionary and reproductive rates and shorter generation times. We chose four *Ambystoma tigrinum* virus (ATV) strains and their sympatric salamander hosts (*Ambystoma tigrinum)* to test for the presence of local adaptation in a fully crossed infection experiment. Infectivity has been the sole measure of local adaptation in most studies though it does not measure pathogen fitness in its complexity. Our study incorporated four measures of pathogen fitness including infectivity, within host growth, virulence, and transmission. We combined two analysis methods to identify local adaptation: "local vs. foreign" and "home vs. away" as both discern local adaptation in unique ways. As host- pathogen interactions vary across space, different patterns of selection can lead to trait divergence and local adaptation. We chose viral strains from different selection regimes based on putative pathogenesis genes to assess the affect the strength of selection had on local adaptation. We predicted that strains under weak selection

would be locally adapted or ahead of their sympatric hosts, while those under strong selection would be locally maladapted or "lagging" behind their sympatric hosts.

Infectivity alone was a misleading determinant of pathogen local adaptation. Nearly all ATV strains appeared locally adapted based on infectivity while other measures of pathogen fitness suggested local maladaptation. When coupled, the two analysis methods gave a clearer picture of the interaction than when used independently. Using these combined analysis methods, we found that the strength of selection appears to affect local adaptation in the predicted manner. The viral strain under weak selection and that under intermediate selection appeared to be locally adapted to their sympatric hosts. The strain under strong selection appeared to be locally maladapted to its sympatric host, which is indicative of the pathogen experiencing a lag as the host is temporarily ahead. Our data capture a specific moment in time of the interaction between ATV strains and their sympatric hosts, so the observed range of states of local adaptation may be an expected outcome.

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Dedication

This thesis is dedicated to my mother who will always be with me in spirit, to my husband who has supported me throughout, and to our unborn child who awakened my powers.

INTRODUCTION

Theory suggests that pathogens should be locally adapted due to higher evolutionary and reproductive rates and shorter generation times than their hosts (Kaltz and Shykoff 1998; Dybdahl & Storfer 2003; Kawecki & Ebert 2004). Locally adapted pathogens are predicted to have higher mean fitness on sympatric versus allopatric host populations, and several transplant experiments in plant and invertebrate systems support this prediction (reviewed in Kaltz & Shykoff 1998; Greischar & Koskela 2007; Hoeksema & Forde 2008). Other investigations, however, showed no relationship between pathogen fitness and degree of host sympatry (Zahn et al. 2002), equivocal results (Briskie et al. 1992; Soler & Soler 1999; McCoy et al. 2002), or apparent "maladaptation" whereby pathogens showed higher performance on allopatric hosts than sympatric hosts (Imhoof & Schmid-Hempel 1998; Kaltz et al. 1999; Oppliger et al. 1999; Greischar & Koskela 2007).

Discrepancies among studies of whether pathogens appear locally adapted may be due to the fact that infectivity (i.e., percent exposed hosts that become infected) is often the sole fitness correlate measured (Dybdahl & Storfer 2003). However, the relationship between infectivity, virulence (defined here as degree of host damage) and transmission ultimately determines pathogen fitness (MacKinnon & Read 1999; Dybdahl & Storfer 2003; Laine 2008). For example, a highly infectious pathogen with low transmission may have low fitness on its host. Alternatively, a highly virulent pathogen may have low transmission and burn itself out quickly (e.g., Ebola). Indeed, transmission may be positively or negatively correlated with infectivity or virulence (e.g., see MacKinnon & Read 1999). Thus, studies that incorporate estimates of all

three components of pathogen fitness in multivariate analyses are necessary to accurately assess pathogen fitness and local adaptation.

Conclusions regarding pathogen local adaptation may also be affected by the way local adaptation is determined. Some researchers have suggested that the typical "home versus away" comparison of sympatric versus allopatric performance is inaccurate (Kawecki & Ebert 2004). Instead, "local versus foreign" comparisons that compare performance of multiple pathogen strains on the same host population have been advocated. Using this approach, locally adapted pathogens are expected to have higher performance on their source host population than pathogens lacking any history of selection in that population (Kawecki & Ebert 2004). Each comparison can be misleading on its own when there are significant main effects of host or pathogen present, thus a clearer picture is provided when both are used.

Spatial variation in strength of selection (i.e., "selection mosaics" *sensu* Thompson 2005) is often expected among host and pathogen populations. The process of coevolution can be observed at the scale of the population, although it is ultimately interactions between individuals that generate reciprocal selection (Thompson 1994, 2005). Furthermore, hosts and pathogens should generally exhibit spatial variation in population genetic structure because environments are patchy and habitat quality varies (Thrall & Burdon 1997; Hochberg & Van Baalen 1998; Hochberg & Holt 2002). Consequently, the strength of coevolutionary interactions should vary spatially, as in the geographic mosaic model (Gomulkiewicz et al. 2000; Nuismer et al. 2000; Brodie et al. 2002; Lively et al. 2004; Forde et al. 2004; Thompson 2005). Indeed, some detailed empirical studies have shown that spatial variation in the strength of selection critically influences coevolutionary dynamics (e.g., Benkman 1999; Brodie et al. 2002; Thompson & Cunningham 2002; Thompson 2005).

The above arguments suggest that studies of pathogen local adaptation should take into account selection history on the pathogen, incorporate multivariate estimates of pathogen fitness, and consider both "home versus away" and "local versus foreign" estimates of local adaptation. A system highly suitable for such studies is that of tiger salamanders (*Ambystoma tigrinum*) and *Ambystoma tigrinum* virus (ATV) throughout Western North America. ATV is in the genus *Ranavirus* in the family *Iridoviridae* and has been implicated in mass epizootics of western North American tiger salamander populations, including the endangered Sonoran subspecies, *A. tigrinum stebbinsi* (Jancovich et al. 1997). Ranaviruses are implicated in amphibian epizootics worldwide, as they tend to be highly virulent and appear to spread with relative ease in aquatic environments or during direct contact (Chinchar 2002; Daszak et al 2003). ATV is monophyletic relative to other ranaviruses (Jancovich et al. 2005), and in most cases, phylogenetic concordance between nodes on salamander and ATV gene trees suggest a strong coevolutionary history (Storfer et al. 2007).

Three host immune evasion genes were sequenced across 19 strains of ATV and spatial variation in the intensity of selection was found (Fig. 1; Ridenhour & Storfer 2008). Viral eif2-α, putatively involved in host interferon downregulation (Essbauer et al. 2001; Majji et al. 2006), was under strong purifying selection across all strains. Figure 1 shows geographic variation in the intensity of selection on two additional host immune evasion genes: 1) β-OH steroid oxidoreductase, putatively involved in host immune suppression via corticosteroid upregulation (Reading et al. 2003); and 2) a CARD-Caspase gene, likely involved in downregulating host cell apoptosis (programmed cell death), thereby increasing cell longevity for virus replication (Bouchier-Hayes & Martin 2002). Involvement of these three genes in pathogenesis has been

verified with knockout experiments, whereby virus strains lacking these genes were less virulent than intact strains (Reading et al. 2003; Majji et al. 2006).

We conducted a fully-factorial experiment to test whether ATV is locally adapted under four different apparent selection regimes: weak selection, intermediate selection, strong selection, and a presumably defunct strain where strength of selection is unknown. We predicted that if spatial variation in selection affects local adaptation, then ATV strains under stronger apparent selection at the three pathogenesis genes would be less well adapted (i.e., lagging behind hosts) than strains under weaker selection. Finally, we compared infectivity alone as a predictor of pathogen local adaptation with estimates of within host growth, transmission, and virulence among ATV strains. We tested whether ATV was locally adapted for all four fitness measures using both home versus away and local versus foreign measures.

MATERIALS & METHODS

Animal Collection and Rearing

Animals were collected as eggs or hatchlings by hand or dip-netting during summer, 2007 from localities where an ATV strain was previously isolated (Fig 1). Collection of eggs occurred haphazardly from at least three different egg masses per locality to minimize confounding effects of common inheritance. *Ambystoma tigrinum diaboli* (DIAB) was obtained through the Bollinger lab at the University of Saskatchewan in Saskatoon, Saskatchewan, Canada, *A. t. melanostictum* (MEL) was obtained in the Targhee National Forest, Teton County, MI , *A. t.*

nebulosum (NEB) was obtained on the Kaibab Plateau, Kaibab National Forest, Coconino County, AZ, and *A. t. mavortium* (MAV) was obtained in the Pike National Forest, Teller County, CO. Upon entry into the lab, animals were kept in an environmental chamber that was held at a constant temperature (20° C) and 12h light: 12 h dark cycle. Animals were individually housed upon hatching in autoclaved clear 24 oz. plastic containers filled with 500mL of water treated with Reptisafe© (Zoo Med Laboratories San Luis Obispo, CA, USA) to remove chlorine and chloramines. Water changes occurred on a weekly basis, at which time individual containers were cleaned and refilled with freshly prepared water. An *ad libitum* feeding schedule commenced with the introduction of laboratory hatched brine shrimp soon after salamander larvae hatched, followed by California blackworms (*Lumbriculus variegates)* when larvae were approximately one month old. Salamanders acclimated in the laboratory for approximately 12 weeks, at which time all individuals across subspecies were at the same life stage (stage #18 from Watson & Russell 2000).

Virus Culture

ATV strains were cultured and titered in *epithelioma papilloma cyprini* (EPC) cells at 20°C using methods in Jancovich et al. (1997, 2001). Growth media included Eagle's Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. No strain was passed more than three times in cell culture to prevent attenuation. The four ATV strains used in the experiment were chosen based on each of the four the selection regimes determined by Ridenhour & Storfer (2008). Each strain will be referenced by the subspecies from which they were isolated from, i.e. DIAB-v, NEB-v, MAV-v, and MEL-v.

Cross-infection Experiment

We used a complete 5 (virus isolates from each selection regime**;** see Fig. 1: MAV-v, MEL-v, NEB-v, DIAB-v + a negative control) x 4 (salamander subspecies sympatric to each viral strain MAV, MEL, NEB, DIAB) factorial design with 30 individuals from each subspecies per treatment, for a total of 600 animals. Individuals were randomly assigned to treatments and placement of individuals was randomized across shelves in the environmental chamber. Prior to infection, animals were weighed and snout-vent (SVL) length was measured. Infection occurred at a concentration of $10^{3.5}$ plaque forming units (PFU) for each viral strain via water bath exposure for one week, sufficient to induce infection in other experiments (see Jancovich et al. 2001; Forson and Storfer 2006). Controls were treated with an equal volume of cell culture growth media via water bath exposure. Controls were kept separate from virus treatments to prevent cross-contamination. The experiment was run for 34 days, or until no individual died for 5d. All surviving animals were euthanized in 2% tricane methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA), at which time they were weighed, SVL was measured, and a tail clip was taken. All tail clips were stored in 95% ethanol.

Animals were checked twice a day for the duration of the experiment, noting initial signs of infection, changes in behavior, or mortality. When mortality occurred, the animal was weighed, SVL was measured, and a tail clip was taken. Infectivity was determined by the percentage of individuals in each treatment that tested positive for viral DNA in the tail clip. Within host growth was determined through viral load in a standardized quantity of DNA extracted from the tail clip. Transmission was estimated as the viral concentration in the water housing the animals, as follows. A water sample was collected from animals during the weekly

water change that occurred at 14 d to estimate transmission between days seven and 14. All of the water that housed the animal was poured into a plastic bag and immediately frozen at -20°C. Water samples were taken upon death for any individuals that died prior to 14d. Virulence was determined by the number of days infected animals remained alive during the experiment. When a host remains alive, the pathogen is presumably able to exploit its resources and continue to create progeny within its host. Five animals were lost during the experiment and all data pertaining to those individuals were left out of all analyses.

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA from tail tissue was extracted using Qiagen DNeasy Blood & Tissue Kits (Qiagen, Valencia, CA, USA) following instructions for animal tissue. Briefly, tissue was lysed overnight at 56°C and spun through columns to separate the DNA, which was then washed and eluted. The amount of DNA in the tail eluate was then quantified with a NanoDrop ND-1000 (Thermo Fisher Scientific Wilmington, DE, USA) and all samples were diluted to 20ng/uL for use in PCR.

DNA from water samples was extracted using QIAamp UltraSens Virus Kits (Qiagen, Valencia, CA, USA) following instructions for blood plasma. Briefly, water samples were completely thawed to 20°C and homogenized by hand. One milliliter was taken from each sample bag to use in the extraction, which underwent a 10 minute lysis step followed by spinning through a column to separate, wash, and elute the DNA.

Quantitative real-time PCR (qPCR) was used to estimate the amount of viral DNA present in both water and tail clips from each animal. A 70-base pair region of DNA from the viral major capsid protein (MCP) was amplified using a forward primer (5’ ACA CCA CCG

CCC AAA AGT AC 3'), a reverse primer (5' CCG TTC ATG ATG CGG ATA ATG 3'), and a labeled fluorescent probe (5' FAM-CT CAT CGT TCT GGC CAT CAA CCA C-TAM 3') following methods in Brunner et al. (2004). Five microliters of DNA template was added to a 20uL mix of Taqman 2X Universal PCR Mastermix (No AmpErase UNG) (Applied Biosystems, Foster City, CA, USA) with 300 nmol forward primer, 900 nmol reverse primer, and 250 nmol probe, and run on an ABI 7300 Thermocycler (Applied Biosystems, Foster City, CA, USA). PCR conditions are as follows: one cycle at 95°C for 10min, and then 40 cycles of the following program: 95°C for 20 sec, 56°C for 20 sec, and 72°C for 30 sec (Forson & Storfer 2006). All qPCR plates were run with a range of pure ATV DNA standards, and data were collected only from plates with a standard curve of $r^2 > 0.98$. Standards were created using a log-based dilution series (from 5×10^8 viral copies/µL to 5×10^2 viral copies/µL for tissue samples and of 5×10^7 viral copies/µL to 5×10^1 viral copies/µL for water). All standards were run in duplicate and unknowns were run in triplicate. If the standard error of any unknown sample was > 0.2, the outlier was removed and viral quantification was estimated by using the remaining two replicates. If this did not produce a standard error <0.2, the sample was rerun.

Statistical Analyses

Two way ANOVAs were used to test the main effects of viral strain, host strain and the interaction on each of three fitness correlates: within host growth, virulence and transmission. Analysis of covariance was initially used to account for the initial differences between in mass among host treatments but no significant effects of mass were found, so two-way ANOVAs were used instead. Infectivity was tested using logistic regressions as they have binomial

distributions. Quantities of viral DNA determined by qPCR were log transformed to meet assumptions of normality for all analyses. For virulence, the inverse of the number of days an individual infected host remained alive during the experiment was used for ANOVAs to meet assumptions of normality. A simple linear regression was used to determine the correlation between within-host growth and transmission. Statistical analyses were conducted using SAS software (SAS Institute, NC 1999) and R 2.8.0 (R Foundation for Statistical Computing 2008).

We also assessed both local versus foreign and home versus away criteria for local adaptation for each of the four fitness correlates as the experimental design is fully reciprocal. Briefly (following Morgan et al. 2005), for each virus strain, the mean trait value across all hosts was subtracted from the value on its local host to determine "home vs. away" (HVA) status of local adaptation for each of the four viral strains. The mean trait values across all virus strains on a single host subtracted from the value of the sympatric virus strain was used to determine "local vs. foreign" (LVF) status of local adaptation for each of the four viral strains. A positive result signaled that a virus strain was locally adapted; conversely, a negative result implied local maladaptation. For each viral strain the results of both HVA and LVF analyses were averaged to determine an overall picture of local adaptation.

RESULTS

Infectivity was assessed after mortality occurred or at the end of the experiment using qPCR on tail tissue. An animal was considered positive when the average quantity of virions detected was above the threshold of the most dilute standard included on the plate, 10^2ng/uL DNA template.

Over eighty-seven percent (87.15%, σ= 16.88) of individuals from treatment groups that received any of the four strains of ATV tested positive for the presence of viral DNA. Variation in the proportion of infected individuals was driven by both host ($G^2 = 23.17$, df = 3, $P < 0.001$; Table 1) and virus ($G^2 = 57.87$, df = 3, $P < 0.001$; Fig. 2). A logistic regression revealed a significant interaction between virus and host ($G^2 = 29.03$, df = 9, $P = 0.006$).

Initial signs of infection were noted as early as 8d post infection, with the first mortality events occurring at 10d. Mortality peaked on day 14 with 44 individuals and tapered off at an average of 2.9 deaths per day after 19d until 29d, the last day that a mortality event occurred. Every individual that died in the infected treatments was found positive for viral DNA via qPCR totaling 54.82% of all infected individuals. One control individual died during the experiment, but was found negative for viral DNA in both the tail tissue and water sample.

Variation in virulence was driven by significant main effects of both host ($F_{3,404} = 15.81$, P = <.0001; Table 1) and virus ($F_{3,404} = 54.58$, P = <0.0001). Host effects were driven by high virulence in DIAB hosts ($\bar{x} = 0.049$, σ= 0.014) and low virulence in NEB hosts ($\bar{x} = 0.039$, σ= 0.009) while all virus strains differed significantly in virulence (Fig. 2) with the extremes of MEL-v ($\bar{x} = 0.060$, σ= 0.007) and NEB-v ($\bar{x} = 0.033$, σ= 0.002). There was no significant interaction between main effects.

Within-host growth was determined by qPCR of a standardized amount of DNA extracted from tail tissue and ranged from 4.03×10^6 viral copies per 20ng/µL DNA (MEL/NEB-v cross) to 3.86×10^7 viral copies per 20ng/µL DNA (DAL/MAV-v cross). Significant main effects of both host ($F_{3,464} = 7.48$, P = <.0001) and virus ($F_{3,464} = 58.83$, P = <.0001) drove variation in within host growth (Table 1). A significant interaction was also present ($F_{9,464}$ =

4.22, P = <.0001). Variation in the hosts was driven by DIAB hosts ($\bar{x}$ = 2.66 x 10^7, σ= 3.62 x 10^6) and NEB hosts ($\bar{x}$ = 1.12 E07, σ= 4.01 x 10^6), who had the highest and lowest levels across all virus treatments, respectively. All viruses were found to have significantly different within host growth quantities (Fig. 2), with MEL-v as the highest ($\bar{x}$ = 2.48 E07, σ= 3.46 x 10^6) and NEB-v the lowest ($\bar{x}$ = 6.87 E06, σ= 4.12 x 10^6).

Transmission was determined by qPCR of a water sample taken at 14d or upon death, if before 14d. Significant main effects of both host ($F_{3,461}$ = 4.37, P = 0.0046; Table 1) and virus ($F_{3,461}$ = 35.66, P = <.0001) drove variation in transmission (Table 1). All virus strains differed significantly in transmission levels (Fig. 2), ranging from MEL-v ($\bar{x}$ =1740.0, σ= 761.44) with the highest quantity and NEB-v with the lowest mean quantity ($\bar{x}$ =31.42, σ= 13.66), which was considered negative as it fell under our lowest qPCR standard of 5 x 10^1 viral copies/µL. NEB hosts were the primary source in variation of hosts with the lowest mean transmission measurement ($\bar{x}$ =120.26, σ= 53.31). A linear regression found a significant correlation between within-host growth and transmission (y = 0.6165x - 1.0357, R^2 = 0.5707, P=<.0001; Fig 5).

The two local adaptation analysis methods, LVF and HVA, revealed different patterns across the four pathogen fitness traits. Within each analysis type, patterns of local adaptation were also varied with no set of fitness traits converging on a clear picture of local adaptation for any one viral strain (Fig 4). Each viral strain appears locally adapted to its sympatric host based on infectivity alone in the HVA and 3 of 4 strains (not NEB-V) appear locally adapted for infectivity in the LVF analysis. When using both the HVA and LVF analysis methods in combination, it appears that NEB-V is strongly maladapted across 7 out of 8 traits and both DIAB-V and MAV-V are possibly locally adapted based on 6 out of 8 traits (Fig 4). MEL-v is balanced between the two at 4 out of 8 traits (Fig 4).

DISCUSSION

Multivariate Measures of Pathogen Fitness

The complex relationship between host and pathogen necessitates a thorough set of measurements to define pathogen performance (MacKinnon & Read 1999; Dybdahl & Storfer 2003; Laine 2008). Our findings suggest that no single variable can be used to independently estimate local adaptation in our system. If infectivity were to be the sole fitness measure used, it would appear that all virus strains perform best in home populations compared to away populations and most of the strains perform better on their local host than do foreign strains (Fig 4). Taken independently, these data suggest that the virus strains are generally locally adapted. However, none of the virus strains consistently show local adaptation across all other fitness measures. For example, NEB-v is locally adapted for infectivity according to HVA analysis while all other fitness traits across both HVA and LVF analyses show the virus is maladapted (Fig 3, Fig 4). MEL-v appears locally adapted for infectivity according to both analysis methods, but is maladapted for all other traits according to HVA analysis. Overall, MEL-v shows an equivocal picture of local adaptation at 4/8 traits (Fig. 4).

The above examples illustrate that multiple measures of pathogen fitness should be estimated to assess local adaptation. Infectivity is a measure of the ability of a pathogen to enter and replicate in exposed hosts, not how successful it is in reproducing or transmitting offspring to new hosts before killing its present host. ATV is a highly pathogenic ranavirus that tends to cause mortality in infected larvae within a few weeks (Chinchar 2002). Despite the appearance of local adaptation according to infectivity, it is arguable that an ATV strain is not locally

adapted if it reproduces in relatively low quantities within its host and has a low transmission rate before killing the host. That is, the strain has burned itself out too quickly. MEL-v performed this way in our experiment. While locally adapted for infectivity in both HVA and LVF, it was maladapted for transmission in both. It was also maladapted across all other fitness traits in HVA. Local adaptation is a continuous trait; our results show that the quantity of virions produced within the host (within host growth), the amount of time the host provided a suitable environment for the virus to reproduce (virulence), and the quantity of virions shed (transmission) should be considered in conjunction with infectivity to provide a complete assessment of pathogen performance. A strong correlation between within host growth and transmission partially supports this claim (Fig. 5).

Since the experiment was fully crossed it was possible to combine both HVA and LVF analysis methods, though the two analysis methods did not converge on a similar picture of local adaptation in every case. The results of three of the viral strains are divided between indicating the presence of local adaptation or that of local maladaptation on their sympatric hosts depending on the analysis method used (Fig 4). For example, DIAB-v performs best on its sympatric host DIAB according to all fitness measurements compared to allopatric hosts (HVA) but when determining performance based on LVF analysis, the results are unclear: DIAB-v appears locally adapted for infectivity and transmission, but not for within-host growth and virulence. A similar scenario is present for MAV-v, except the LVF analysis shows clear local adaptation while the HVA analysis has mixed results. We chose to combine the two methods to get an overview of local adaptation, therefore there is a strong suggestion that both MAV-v and DIAB-v are locally adapted by a majority of traits (6/8; Fig. 4).

In future studies, experimental designs should facilitate analysis of all fitness variables simultaneously to estimate a single measure of pathogen fitness. However, the nature of the data we collected made this difficult. For example, we only estimated transmission at a single point in time. It would be beneficial to take multiple measurements so a rate of transmission could be calculated. A similar scenario could be used for within host growth.

Selection Mosaic Effects on Local Adaptation

A foundation of the geographic mosaic of coevolution is that local systems will reveal different combinations of traits or genotypes as patterns of selection will differ at the local scale (Thompson 1994, 1999). Ridenhour & Storfer (2008) revealed a mosaic of selection across ATV strains based on two genes putatively involved in viral pathogenesis. Both genes appear to be under strong selection in NEB-v which in turn appears to be locally maladapted (7/8 traits; Fig. 4). In addition, we found NEB-v to be a relatively "weak" strain with the lowest mean infectivity, within host growth, virulence, and transmission measurements (Fig. 2). Maladaptation on a sympatric host is an occasional outcome of host-pathogen interactions as lags are experienced due to asynchronous cycling of gene frequencies as parasites track host genotypes (Gandon et al. 1996; Morand et al. 1996; Thompson 1999; Lively 1999; Nuismer 2006). The detection of strong selection in NEB-v on putative pathogenesis genes may suggest that NEB-v is currently behind its NEB host and experiencing a lag. In general, the NEB host appeared more resistant comparatively across most other viral strains (Fig. 3), reinforcing the idea that it may currently be ahead of the virus. Alternatively, the DIAB-v strain appears to be locally adapted (6/8 traits; Fig. 4) and is under comparatively weak selection (no selection on

either gene). At this moment in time, the DIAB-v pathogen could be considered ahead of the host. The MAV-v strain shows a similar local adaptation pattern to DIAB-v (6/8 traits; Fig. 4) but only one of the two genes is under strong purifying selection. The strength of selection on MEL-v is unknown due to frameshift mutations in both genes. One assumption is that these frameshift mutations would make MEL-v "defunct" (Ridenhour & Storfer 2008). However, our experiment revealed that MEL-v is a particularly virulent strain that tends to infect close to 100% of its hosts, kill its hosts quickly, and have high viral loads in both water and tissue. These results clearly show that the frameshift mutations do not affect the strain's ability to infect and perhaps facilitate the strain's high fitness. Lively (1999) found that higher rates of virulence tended to culminate in similarly high rates of local adaptation, but neither is this the case for MEL-v. MEL-v has an overall equivocal pattern (4/8 traits) but appears locally adapted (3/4 traits) in our LVF analysis and maladapted (1/4 traits) in our HVA analysis (Fig. 4). It is interesting to note that MEL-v is maladapted for transmission in both analysis methods and is therefore killing its host too quickly to achieve optimal transmission.

Fluctuations in selection in host-pathogen interactions are expected (Thompson 1999, Lively 1999, Nuismer 2006). Our data capture a specific moment in time of the interaction between ATV strains and their sympatric hosts, so it is perhaps to be expected that we will observe a range of states of local adaptation. There is a strong suggestion of a correlation between viral strain selection regimes and whether or not the strain appears locally adapted at this moment in time. This picture is subject to change in any given generation depending on current selection pressures.

Pathogen Driven Variance

Our analyses revealed that significant differences among virus strains drove variation across all fitness variables (Table 1; Fig 2). The MEL-v strain, as previously noted, had consistently high means across all response variables, while NEB-v consistently had low means. In addition, all virus strains were significantly different across most fitness traits (Fig. 2). Infectivity was the only measurement that was an exception as MEL-v and MAV-v were not significantly different. These data are not surprising in light of the mosaic of selection revealed on putative pathogenesis genes. It is interesting to note that although there was a main effect of host found across all variables, it was generally driven by only one or two of the hosts (i.e. the others were not significantly different from each other; data not shown). For example, NEB was the only host that was significantly different from others for the transmission measurement and NEB and DIAB were the hosts that drove significance for the within host growth measurement (data not shown). A possible implication of these data is that overall most of the hosts mount a similar defense to infection. NEB is always an exception and appears to be temporarily ahead of its sympatric pathogen strain which may explain the consistent significant differences from other hosts.

The immune system of Ambystomatid salamanders is not well characterized (Carey et al. 1999) and shows an apparent lack of adaptive immunity (Cotter et al. 2008). Adaptive immunity is the classic defense against a recognized pathogen and enables a host to mount a more robust defense upon re-infection. ATV is known for epizootics where mortality can reach up to 90% or more (Chinchar 2002; Daszak et al. 2003), and mass die-offs have been noted on the Kaibab Plateau of AZ (where NEB hosts and NEB-v originated) in cycles. Metamorphosed hosts can

harbor subclinical infections that lead to re-introduction of ATV and the subsequent infection of the next generation of larvae when hosts return to breeding sites (Brunner et al 2004). These patterns of cycling with regular host die-offs occurring reinforce the idea that there is a lag seen in NEB-v that lead to current local maladaptation. The long term ecological data on infection cycles are not available for the other host-pathogen combinations we used, but it is reasonable to expect similar cycles to be present based on patterns of local adaptation that we found in other hosts.

CONCLUSION

Our study suggests that host- pathogen interactions are too complex for any one measure of pathogen fitness to be an accurate predictor of local adaptation. No single measure of pathogen fitness in this system can reliably discern local adaptation; it is the combined effect of many fitness measures and the utilization of two analysis methods that help to elucidate the relationship between host and pathogen. Each method of analysis tells a different story about pathogen local adaptation and each has its benefit (Kawecki & Ebert 2004). In addition, our experimental design was fully crossed and conducive to the combination of these analyses. We defined local adaptation as both higher performance on sympatric hosts than across allopatric hosts and higher performance on sympatric hosts than across allopatric pathogens. That is, a locally adapted pathogen always performs best on its sympatric population. This allowed us to fully evaluate whether or not a pathogen strain was locally adapted while avoiding the confounding effects that can occur when main host and pathogen effects are present.

The nature of our data provided us with a snapshot of the host-pathogen interaction. We found that spatial variation in selection does have an effect on local adaptation as predicted. The

strain under strong selection (NEB-v) was maladapted where the host appears temporarily ahead of the pathogen, the strains under weak (DIAB-v) and intermediate (MAV-v) selection appeared locally adapted to their sympatric hosts, while the unique strain with frameshift mutations (MEL-v) had an equivocal picture of local adaptation. Due to the fluctuations in selection between hosts and pathogens, it is perhaps not surprising that a snapshot reveals the pathogen strains are in a range of states of local adaptation. Additional genomic data for both pathogens and hosts across entire genomes would elucidate the strength of selection pressures that the host and pathogen exert on each other. It would be interesting to examine different snapshots of each interaction to compile a better picture of the oscillations in gene frequencies over time.

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FIGURE LEGENDS

Figure 1. Map of collection sites for each of the four viral strains and their sympatric hosts. Colors correspond to variation in selection on the two genes putatively involved in ATV pathogenesis (Ridenhour & Storfer 2008): DIAB-v: blue, weak selection; MAV-v: red, intermediate selection; MEL-v, green, unknown selection; NEB-v: purple, strong selection Each viral strain is named after its host strain.

Figure 2**.** Means of viral performance on each fitness trait. Letters above bars indicate significant differences of means. Colors correspond to the relative strength of selection on each strain as estimated in Ridenhour and Storfer (2008): DIAB-v: blue, weak selection; MAV-v: red, intermediate selection; MEL-v, green, unknown selection; NEB-v: purple, strong selection. A. Infectivity: calculated by percent infected B. Virulence: the inverse of days alive infected C. Within Host Growth: viral load in tissue, log transformed D. Transmission: viral load in water, log transformed.

Figure 3. Viral performance across four fitness traits on all host populations (lines). Colors correspond to the relative strength of selection on each strain as estimated in Ridenhour and Storfer (2008): DIAB-v: blue, weak selection; MAV-v: red, intermediate selection; MEL-v, green, unknown selection; NEB-v: purple, strong selection. Triangles indicate the performance of each strain on its sympatric host. A. Infectivity: calculated by percent infected B. Virulence: the inverse of days alive infected C. Within Host Growth: viral load in tissue, log transformed D. Transmission: viral load in water, log transformed.

Figure 4. Diagram of two methods of determining local adaptation. The top row represents the results of "home vs. away" analysis and the bottom row represents "local vs. foreign" analysis. Light-colored boxes imply that the virus is locally adapted for this trait (higher trait value on sympatric host than mean across other hosts or other viral strains). Dark colored boxes imply that the virus strain is maladapted for this trait (lower trait value on sympatric host than mean across other hosts or other viral strains). I = infectivity, G = within host growth, T = transmission, V = virulence.

Figure 5. Relationship between within host growth and transmission measurements as determined by a log linear analysis. Values are determined by quantitative real-time PCR on host tissue for within host growth and on water samples that the host was housed in for transmission. The relative quantity of viral DNA copies were log transformed.

TABLE 1. Statistical summary of two-way ANOVA results testing the main effects of *A. tigrinum* subspecies hosts and ATV viral strains and their interactions. a. Within host growth, viral load in host tissue. Quantities were log transformed to meet assumptions of normality. b. Transmission, viral load in water at day 14. Water samples were taken upon death for individual hosts that dies prior to day 14. Quantities were log transformed to meet assumptions of normality. c. Virulence, the inverse of days alive infected. d. Infectivity, based on presence of viral DNA in tissue above detection threshold. Data are binomial, and therefore a logistic regression was used to find effects of host and virus. Observed-data likelihood ratio statistics are presented.

a. WITHIN HOST GROWTH

Source	**df**	**MS**	**F-Value**	**Pr > F**
Host	3	28.324	7.48	<0.0001
Virus	3	221.100	58.38	<0.0001
Host * Virus	9	15.967	4.22	<0.0001
Error	452	3.787		

b. TRANSMISSION

Source	**df**	**MS**	**F-Value**	**Pr > F**
Host	3	8.124	4.37	0.0048
Virus	3	66.292	35.66	<0.0001
Host * Virus	9	3.431	1.85	0.0583
Error	449	1.859		

c. VIRULENCE

Source	**df**	**MS**	**F-Value**	**Pr > F**
Host	3	0.0037	15.81	<0.0001
Virus	3	0.0180	54.58	<0.0001
Host * Virus	9	0.0002	0.59	0.8037
Error	391	0.0003		

d. INFECTIVITY

Source	**Deviance**	**df**	**Chi-Square**	**Pr > ChiSq**
Intercept	358.176			
Host	335.010	3	23.17	<0.0001
Virus	227.142	3	57.87	<0.0001
Host * Virus	248.116	9	29.03	0.0006

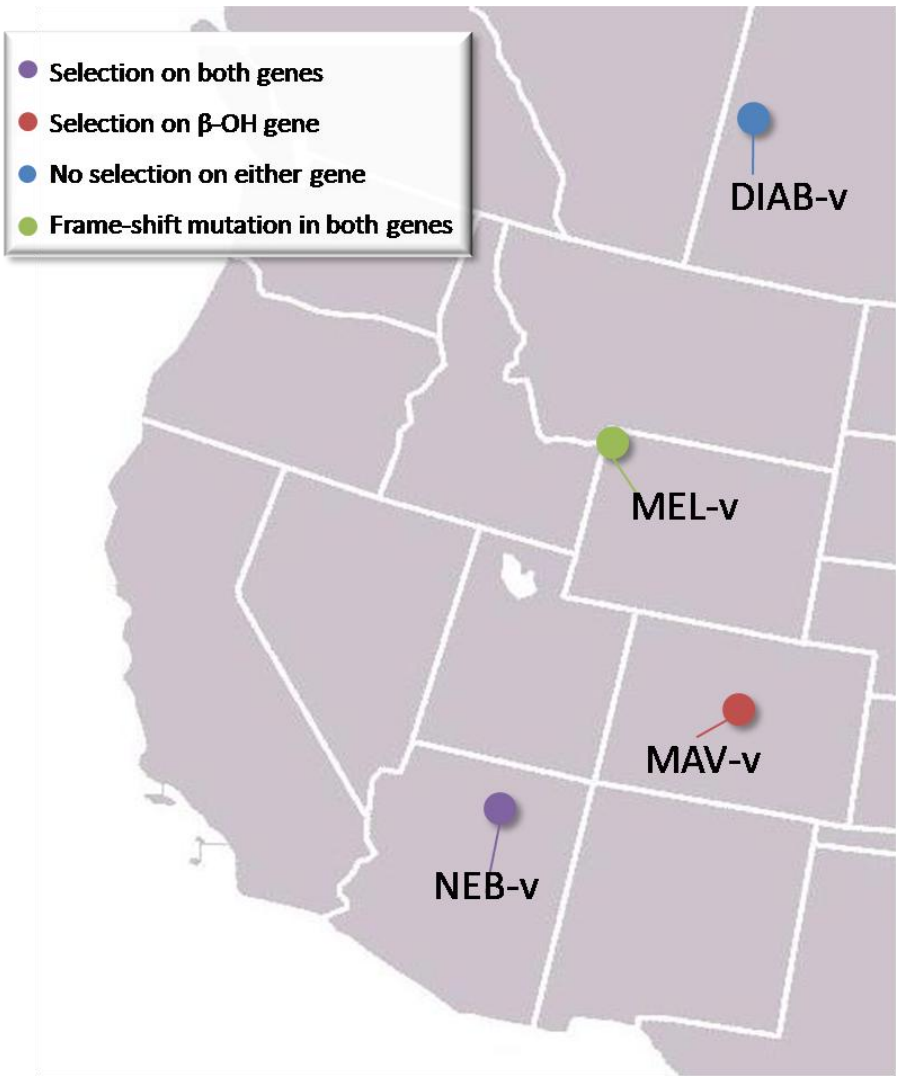
Selection on both genes
Selection on β-OH gene
No selection on either gene
Frame-shift mutation in both genes
DIAB-v
MEL-v
MAV-v
NEB-v

Figure 1

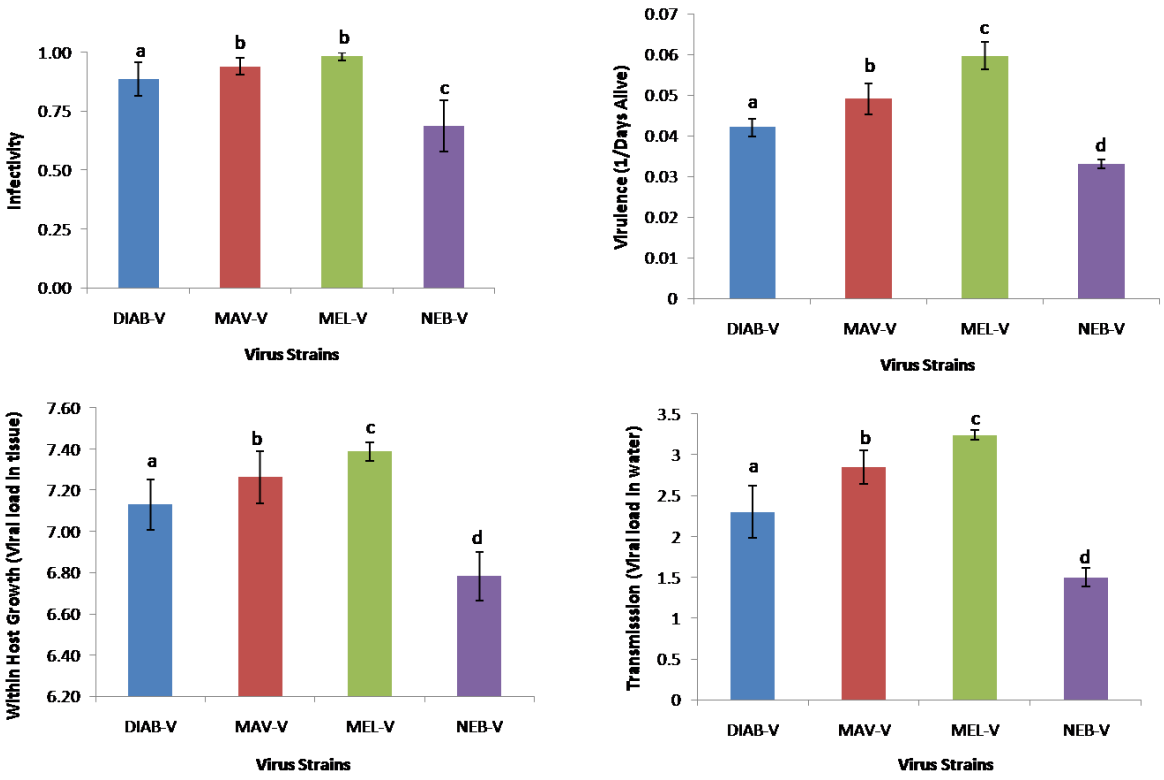
Infectivity
1.00
0.75
0.50
0.25
0.00
a
b
b
c
DIAB-V
MAV-V
MEL-V
NEB-V
Virus Strains
Virulence (1/Days Alive)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
a
b
c
d
DIAB-V
MAV-V
MEL-V
NEB-V
Virus Strains
Within Host Growth (Viral load in tissue)
7.60
7.40
7.20
7.00
6.80
6.60
6.40
6.20
a
b
c
d
DIAB-V
MAV-V
MEL-V
NEB-V
Virus Strains
Transmission (Viral load in water)
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
d
DIAB-V
MAV-V
MEL-V
NEB-V
Virus Strains

Figure 2

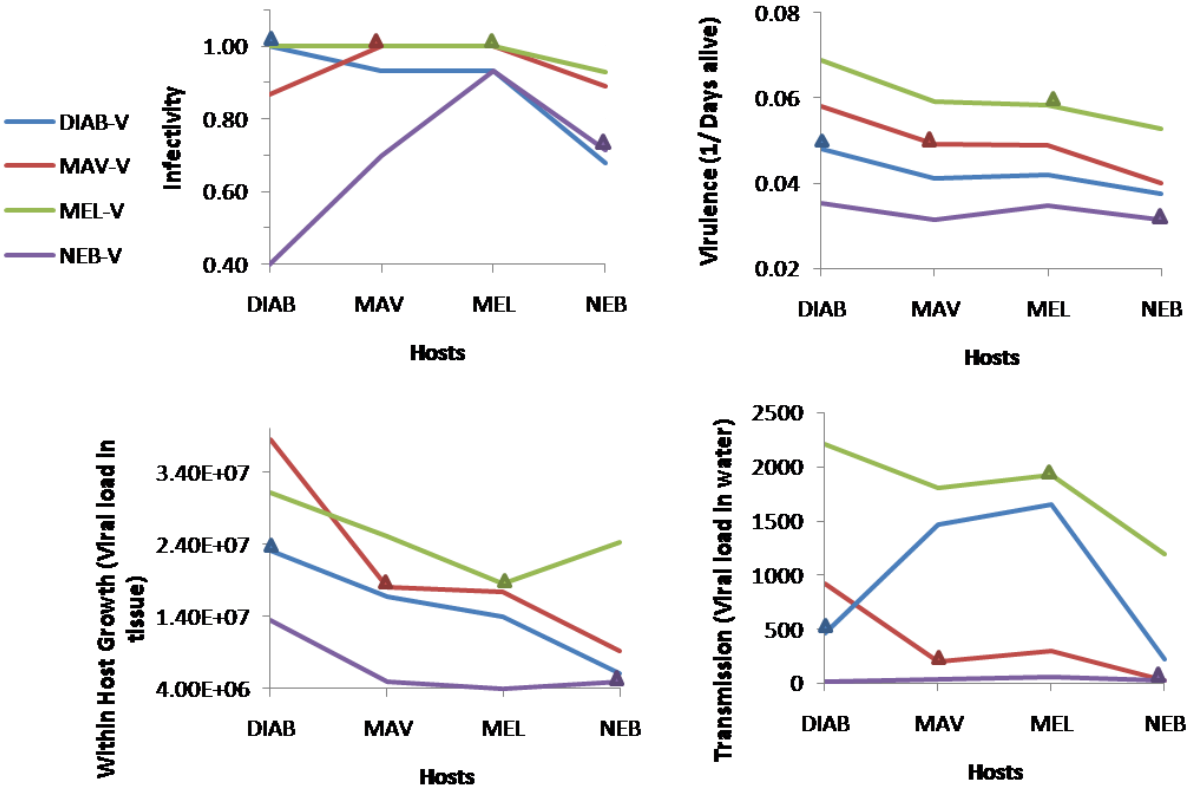
DIAB-V
MAV-V
MEL-V
NEB-V
Infectivity
1.00
0.80
0.60
0.40
DIAB
MAV
MEL
NEB
Hosts
Virulence (1/Days alive)
0.08
0.06
0.04
0.02
DIAB
MAV
MEL
NEB
Hosts
Within Host Growth (Viral load in tissue)
3.40E+07
2.40E+07
1.40E+07
4.00E+06
DIAB
MAV
MEL
NEB
Hosts
Transmission (Viral load in water)
2500
2000
1500
1000
500
0
DIAB
MAV
MEL
NEB
Hosts

Figure 3

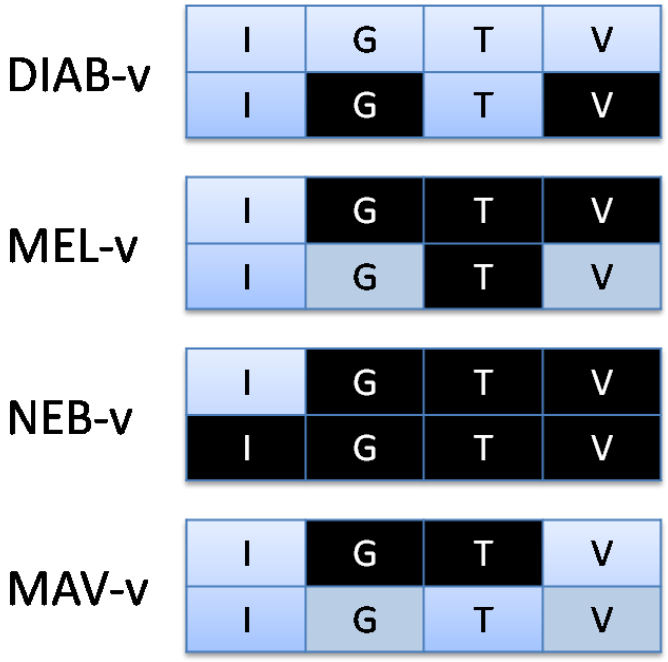

Figure 4

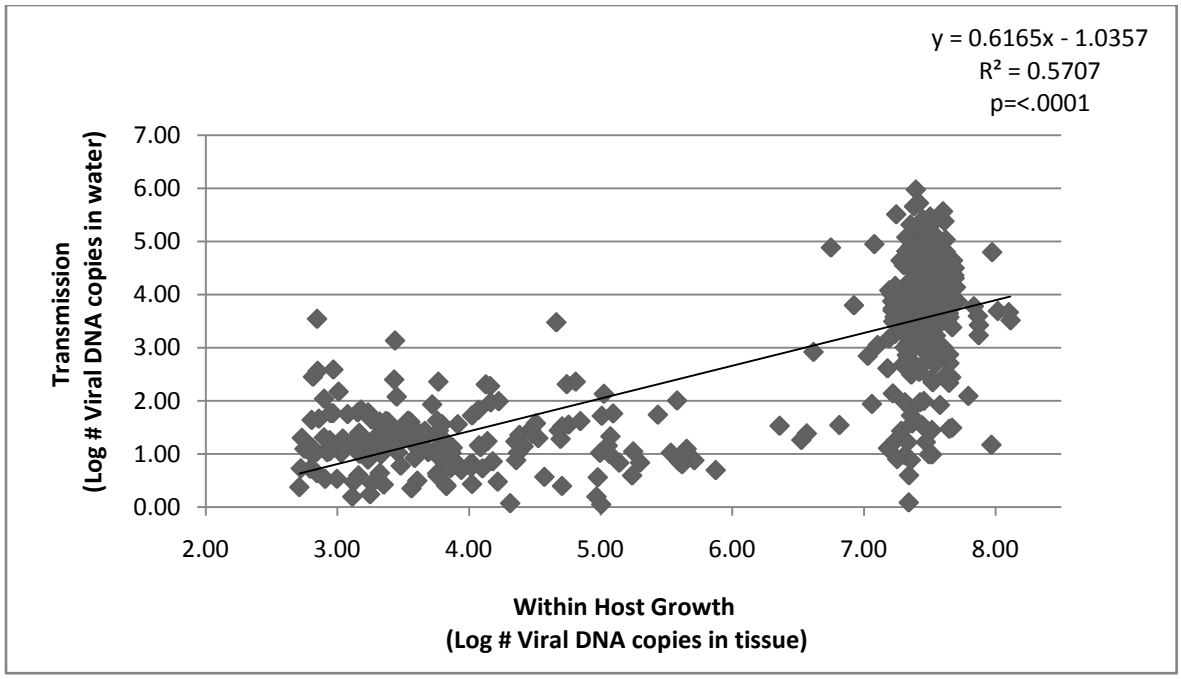
y = 0.6165x - 1.0357
R² = 0.5707
p=<.0001
Transmission
(Log # Viral DNA copies in water)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
Within Host Growth
(Log # Viral DNA copies in tissue)

Figure 5