GENOMIC RESPONSES OF AMBYSTOMATID SALAMANDERS TO INFECTION WITH AN EMERGING VIRUS

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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

AUGUST 2008

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of JENNIFER DIANE STEWART find it satisfactory and recommend that it be accepted.

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Chair

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ACKNOWLEDGMENT

Thank you to my advisor Andrew Storfer for his belief in my abilities as a scientist and for always pushing me to be better. Thank you also to Mark Dybdahl and Doug Call for serving on my committee and giving me their time and honest opinions.

Thank you to Jonathan Eastman for exceptional assistance with statistics and maintenance of sanity, as well as for teaching me to climb and listening to me in innumerable times of trouble. Also to Jacob Kerby for help with experimental design and animal surgery, and for mentoring me like crazy. I would also like to acknowledge Jason Baumsteiger, Karen Benyo, Rebekah Featherkile, Ashley McCally, Melanie Murphy, and Stephen Spear for help with animal care and set up and takedown of experiments – and also for their camaraderie over the years.

Thanks to S. Randal Voss for his assistance and collaboration with chapter one, and for his generosity and hospitality. I'm proud to be an adoptive member of the Voss clan, even if only for a week. Thanks to Robert B. Page for assistance – and more than assistance - in analyzing microarray data and writing chapter one for publication, and to Christopher K. Beachy for experimental design contributions. Additionally I thank John Walker, James Monaghan, and Jeremiah Smith for assistance with RNA extraction, reverse transcriptase PCR, and animal rearing.

A final thank you to Brian Whitmire for assistance above and beyond the call of duty regarding real time and reverse transcriptase PCR. I'll always vortex, Brian.

GENOMIC RESPONSES OF AMBYSTOMATID SALAMANDERS TO INFECTION WITH AN EMERGING VIRUS

Abstract

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August 2008

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Emerging infectious diseases (EIDs) pose a serious threat to the health, stability, and persistence of human and wildlife populations. While several EIDs in human and commercial animal systems are becoming better understood all the time, our knowledge in wildlife disease research needs to be improved for management and conservationthat . In particular, very little is known about the immunological responses of amphibians to pathogens that are causing global population declines. We used a custom microarray gene chip to characterize gene expression responses of *Ambystoma mexicanum* to an emerging viral pathogen, *Ambystoma tigrinum* virus (ATV).

Although *A. mexicanum* appears to have a strong innate immune response, we did not observe gene expression changes that indicate a T cell response, which is associated with clearance of Frog 3 iridovirus in adult *Xenopus*. ATV may be especially lethal to *A. mexicanum* and tiger salamanders because they lack a sufficient cell mediated immune response that is necessary to clear ATV.

Additionally, we performed a 4x4 factorial infection experiment to investigate signatures of local adaptation in this host-pathogen system, using the natural host *Ambystoma tigrinum* and sympatric viral strains that have been demonstrated to be under different selective regimes. Variable selection on the virus across space led to the hypothesis that a virus with particular selection, specifically positive selection, would be

less likely to be locally adapted than one that lacks this selection. We examined within-host viral growth as a proximate measure of viral fitness. The strain under positive pressure appears to not be locally adapted, as would be predicted. However, there was no clear pattern as another virus with positive selection on a virulence gene appeared more locally adapted, although not statistically significantly.

While understanding EIDs is important in all systems, diseases have been implicated as a leading cause of the global decline of amphibians. Learning more about the host genomic responses to this virus will begin to elucidate why this virus is so virulent to its host, and understanding local adaptation of this virus will inform regarding the possibilities of future spread of this highly virulent amphibian pathogen.

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DEDICATION

This thesis is dedicated to my family, who always told me I could be whatever I wanted to be when I grew up.

And also to one anonymous individual, who pulled me out of the darkest time of my life and showed me the light.

Transcriptional response of Mexican axolotls to *Ambystoma tigrinum* virus (ATV) infection

Abstract

Very little is known about the immunological responses of amphibians to pathogens that are causing global population declines. We used a custom microarray gene chip to characterize gene expression responses of axolotls (*Ambystoma mexicanum*) to an emerging viral pathogen, *Ambystoma tigrinum* virus (ATV).

At 0, 24, 72, and 144 hours post-infection, spleen and lung samples were removed for estimation of host mRNA abundance and viral load. A total of 158 up-regulated and 105 down-regulated genes were identified across all time points using statistical and fold level criteria. The presumptive functions of these genes suggest a robust innate immune and antiviral gene expression response is initiated by *A. mexicanum* as early as 24 hours after ATV infection. At 24 hours, we observed transcript abundance changes for genes that are associated with phagocytosis and cytokine signaling, complement, and other general immune and defense responses. By 144 hours, we observed gene expression changes indicating host-mediated cell death, inflammation, and cytotoxicity.

Although *A. mexicanum* appears to mount a robust innate immune response, we did not observe gene expression changes indicative of lymphocyte proliferation in the spleen, which is associated with clearance of Frog 3 iridovirus in adult *Xenopus*. We speculate that ATV may be especially lethal to *A. mexicanum* and related tiger salamanders because they lack proliferative lymphocyte responses that are needed to clear highly virulent iridoviruses. Genes identified from

this study provide important new resources to investigate ATV disease pathology and host-pathogen dynamics in natural populations.

Introduction

Emerging infectious diseases (EIDs) pose a serious threat to the health, stability, and persistence of human and wildlife populations (Berns and Rager 2000, Rachowicz et al. 2005, Daszak et al. 2000, Hudson et al. 2000). Genetic and genomic tools have been incredibly useful for discovery of genes associated with host response and variation in resistance or susceptibility to a variety of pathogens (Scherbik et al. 2007, Glass and Jensen 2007, Tumpey et al 2004). The advent of genomic tools such as microarray analysis has offered new insights into host-pathogen systems. Additionally, their application to genomic response to host disease response allows rapid characterization of candidate genes for further research into control and eradication methods.

EIDs are considered a cause of the global decline of amphibians and two pathogens in particular, *Batrachochytrium dendrobatidis* and Ranaviruses have been implicated in worldwide epizootics (Daszak et al. 1999). Although studies are beginning to investigate possible mechanisms of resistance to these pathogens (Woodhams et al. 2006), in general, very little is known about the immune response of amphibians to EIDs. This is because most natural amphibian species are not used as laboratory models and we lack fundamental molecular tools to investigate disease pathology and host-pathogen interactions at the molecular level for all but a few species (e.g., *Ambystoma tigrinum spp., Xenopus spp.*).

Over the last 15 years, *Ranavirus* infections have been associated with marked increases in morbidity and mortality in fish, reptiles, and amphibians (Chinchar et al. 2002). Ranaviruses are globally-distributed double-stranded, methylated DNA viruses of fish, amphibians and

reptiles and are implicated in amphibian epizootics worldwide (Chinchar et al. 2002, Collins et al. 1988, Cunningham et al. 1996). Both encapsulated and non-encapsulated forms can be infectious. The virus enters the cell via receptor mediated endocytosis or via fusion with the plasma membrane; and DNA and RNA synthesis occur in the nucleus, while protein synthesis occurs at morphologically specific assembly sites in the cytoplasm (Chinchar et al. 2002). In North America, ranaviruses have been isolated from the majority of recently documented amphibian epizootics (Green et al. 2002), including from tiger salamander (Ambystoma tigrinum) epizootics in Saskatchewan, Canada (Bollinger et al. 1999), Arizona (Jancovich et al. 1997), North Dakota, Utah, and Colorado, USA (Jancovich et al. 2005, Docherty et al. 2002). The viral variant that infects tiger salamanders, ATV, is transmitted either via direct contact with an infected animal or immersion in water that contains virus; infected individuals exhibit systemic hemorrhaging, edema, ulceration, and necrosis of the integument and internal organs (Bollinger et al. 1999, Jancovich et al. 2001, Brunner et al. 2005). In cases where ATV infection leads to mortality, it usually occurs within 2-3 weeks of exposure, with animals displaying symptoms often between 8-10 days post-exposure. Thus, ATV can rapidly overwhelm the tiger salamander immune response. However, mortality is not always a pathological endpoint because virulence and resistance are known to vary among ATV strains and tiger salamander populations, respectively, as indicated by both laboratory experiments and field observations (Storfer et al. 2007). Research characterizing the tiger salamander genomic response to ATV is needed to better understand the pathology, virulence, and possible mechanisms of resistance to this emerging disease.

The tiger salamander species complex includes *A. mexicanum* (Mexican axolotl), a model organism with a growing genomic and informatics resource base (Putta et al. 2004). The immune

system of the Mexican axolotl has been extensively studied using several classical approaches. Relative to other vertebrate models, the axolotl immune response has been described as immunodeficient (Kaufman et al. 1995, Tournefier et al. 1998). There are several reasons for this characterization, including: production of only two immunoglobulin (Ig) classes, only one of which regulates the humoral response and neither of which is anamnestic (Fellah and Charlemagne 1998, Tournefier et al. 1998); no response to soluble antigens (Charlemagne and Tournefier 1977); poor mixed lymphocyte reactions (Kaufman et al. 1990, Koniski and Cohen 1992); and lack of cellular cooperation during the humoral immune response as indicated by enhanced humoral immunity following thymectomy or X-ray irradiation (Charlemagne 1979, Charlemagne 1991). Weak immune responses are known for salamanders in general, and the Mexican axolotl and related tiger salamanders are especially susceptible to ATV infections with high observed mortality rates both in the laboratory and in the field. Indeed, an outbreak of ATV in 2003 at the Indiana Axolotl Colony significantly reduced adult stocks before the virus was contained. By way of comparison, adult *Xenopus* effectively clear closely related FV3 *Ranavirus* with an immune response that includes an early T-cell proliferative phase in the spleen (Morales and Robert 2007).

To further investigate the axolotl immune response to ATV, we used an Affymetrix custom microarray to identify genes that were significantly, differentially expressed in the spleen. We then compared these genes to a list of genes associated with regeneration that were previously identified from *A. mexicanum* using the same microarray platform. We reasoned that such a comparison would allow us to filter gene expression responses of humoral cells induced generally in response to injury and stress from those expressed specifically in response to ATV infection. Also, this comparison would potentially identify gene expression signatures associated

with cell proliferation in response to ATV, as we have previously identified many cell proliferation probe sets on the *Ambystoma* genechip that are differentially regulated during spinal cord regeneration (Monaghan et al. 2007). The genes that we describe provide mechanistic insights and new tools to investigate salamander antiviral responses in the laboratory and in natural populations.

Methods

Animal care and surgery protocols

Inbred *A. mexicanum* eggs from a single full-sib mating were obtained from the Ambystoma Genetic Stock Center at the University of Kentucky. Each *A. mexicanum* egg and larva was reared in an individual container in aquifer water treated with ReptiSafe and changed weekly. Individuals were fed brine shrimp *ad libitum* for the first four weeks post-hatching and blackworms (Tubifex) *ad libitum* thereafter. Animals were reared in an environmental chamber on a 12:12 h light:dark cycle at 20ºC. At 4.5 months of age, 12 individuals were injected with 100µl of 10^6 p.f.u./ ml of ATV isolated from the axolotl colony and suspended in cell culture medium. This amount of virus was determined to be the minimum lethal dose via injection in previous unpublished experiments (Storfer, unpublished data) and the strain utilized in the experiment was extracted from axolotls that had previously been infected and killed by the virus. Simultaneously, four uninfected (control) individuals were sacrificed in MS222 for spleen and lung removal. Spleens from all animals were flash frozen in liquid nitrogen. The same surgical procedure was performed on four infected individuals following 24, 72 and 144 hours of infection. Spleen tissue was utilized due to its previously noted importance in CD8+ T cell

immune responses to Ranaviruses, particularly FV3, in frogs (Morales and Robert 2007). Additionally, spleen is an important immune organ as antigens from the blood are processed in the spleen. Lung tissue was removed for viral quantification as it is an internal organ that can be utilized in early stage virus quantification (Stewart, unpublished data).

During the infection period behavioral observations were taken opportunistically. Total RNA was extracted from spleen with TRIzol (Invitrogen) according to the manufacturer's protocol. RNA isolations were further purified using RNeasy mini columns (Qiagen). The amount of RNA present in each isolate was estimated via UV spectrophotometry, and RNA quality was inspected via a 2100 Agilent Bioanalyzer. Sixteen high quality isolates (four replicates at each of four sampling times: 0 (controls), 24, 72, and 144 hours post-infection) were used to make individual-specific pools of biotin labeled cRNA probes. Each of the 16 pools was then independently hybridized to an Amby_001 custom Affymetrix GeneChip (for a more detailed description of the microarray platform see Monaghan et al. 2007 and Page et al. 2007). The University of Kentucky Microarray Core Facility generated cRNA probes and performed hybridizations according to standard Affymetrix protocols.

Quality Control and Data Processing

All quality control and processing analyses were done in R (Ihaka and Gentleman 1996, www.r-project.org). We used the Bioconductor package "affy" (www.bioconductor.org) to perform several quality control analyses at the individual probe level (Bolstad et al. 2005a, Bolstad et al. 2005b). These analyses included: (1) viewing images of the log(intensity) values of the probes on each GeneChip to check for spatial artifacts, (2) investigating measures of central tendency and dispersion by viewing box-plots and histograms of all the GeneChips, (3) viewing pair-wise M versus A plot matrices for replicate GeneChips, and (4) viewing an RNA

degradation plot (Bolstad et al. 2005b) that enables the visualization of the 3' labeling bias associated with all GeneChips simultaneously. Upon conducting these probe level analyses, we background corrected, normalized, and summarized all sixteen GeneChips using the Robust Multi-array Average (RMA) algorithm (Irizarry et al. 2003). Following this, we calculated correlation matrices for replicate GeneChips (four correlation matrices with four GeneChips per matrix; all r from replicate GeneChips > 0.980) on the summarized probe-set level data. The strong correlations observed between replicate GeneChips suggests that we were able to obtain a high degree of repeatability within treatments.

Data Filtering

Microarrays may not accurately quantify the abundance of minimally expressed genes (Draghici et al. 2006). Calculating statistical tests for such genes adds to the multiple testing burden that is inherent to microarray studies. To address this issue, we filtered genes whose mean intensity across all 16 GeneChips was greater than the mean of the lowest quartiles (25^{th} percentiles) across all GeneChips (n = 16, mean = 5.83, SD = 0.06; data presented on a $\log_2$ scale). Upon imposing this filtering criterion, 3619 probe-sets were available for significance testing.

Identifying Differentially Expressed Genes

We used the Bioconductor package LIMMA (Smyth 2004, Smyth 2005) to generate moderated t-statistics for all six of the possible pair-wise contrasts of the four sampling times investigated in our study. LIMMA employs an empirical Bayes methodology that effectively shrinks the sample variances towards a pooled estimate. This approach reduces the likelihood of obtaining large test statistics due to underestimation of the sample variances. The moderated t-

statistics generated by LIMMA test the null hypothesis that the difference between the two groups being compared is zero (*i.e.,* group 1 – group 2 = 0). LIMMA also generates moderated *F*-statistics that test the null hypothesis that none of the contrasts within a family of contrasts are statistically significant. We corrected for multiple testing by applying the step-up algorithm (Benjamini and Hochberg 1995) to the *P*-values of the moderated *F*-statistics associated with our six contrasts. Upon correcting for multiple testing, we identified 2322 genes (probe-sets) that were statistically significant. To prioritize amongst differentially expressed genes, we focused on probe-sets that exhibited two-fold or greater changes at any time-point relative to controls. Any gene that was non-significantly down-regulated but significantly up-regulated at one or more time points was considered up-regulated, and vice versa for classification of up- versus down-regulation. We also required that these probe sets have moderated *F*-statistics greater than or equal to the 50^{th} percentile of the 2322 *F*-statistics from the statistically significant probe-sets ($F \geq 12.68$). We further limited our analysis to only those probe sets that exhibited significant sequence identity with a human reference sequence. We note that 263 probe-sets with no functional annotation were statistically significant, differentially expressed by ≥ two-fold, and had *F*-values ≥ 12.68.

Clustering

Hybridization intensities were averaged within treatment groups (0, 24, 72, and 144 hrs post-infection) and $\log_2$ ratios were calculated for each non-zero sampling time relative to 0 hours post-infection. Genesis v. 1.6.0 (Sturn et al. 2002, Sturn 2000) was used to cluster the $\log_2$ ratio data and to generate heat maps. Clustering was conducted using a Self Organizing Map (SOM) algorithm. Default conditions were used with the exception that the SOM was allowed to

run for 263,000 iterations. The dimensions of the final SOM are 2_x*1_y. These dimensions were determined by comparing output from several different combinations.

Enrichment Analyses

Functional annotation of genes by gene ontology was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, Dennis et al. 2003). Functional annotation clustering was performed using the default settings with the exception of using the highest classification stringency.

Quantitative real-time PCR

We used quantitative real-time PCR (qPCR) to confirm the results of the microarrays. We estimated a fold change for 24 and 72 hr time points using the ΔΔct method of relative quantification (Livak and Schmittgen 2001), utilizing ribosomal protein L 19 as an endogenous control gene. The same total RNA that was used for microarray analysis was used to create cDNA for qPCR using the BioRad iScript cDNA synthesis kit, following manufacturer instructions. Primers for the qPCR were designed using Primer Express 2.0 (Applied Biosystems). Primers were designed to encompass the sequence of GeneChip probe sets (Appendix A). qPCR was accomplished using SYBR Green chemistry.

To verify that exposed animals were infected and to quantify viral load and replication over time, we performed qPCR on lung tissue with TaqMan chemistry following the protocol detailed in (Forson and Storfer 2006). ANOVA with a Tukey's HSD correction for all pairwise comparisons was performed to determine if viral loads were significantly different across time points.

Results

Viral load and disease pathogenesis

Viral load for each animal was estimated using qPCR and then averaged for each time point (Fig 1). The significant increase in viral load across time points indicates that animals were infected and that viral replication was occurring. ANOVA with a Tukey's correction for multiple comparisons confirmed that viral load increased linearly between 24, 72, and 144 hours post-infection, and all time points were significantly different from all other time points ($F_{3, 44} = 242.56$; $P \leq 0.01$).

No animals displayed any gross symptoms of ATV infection in terms of hemorrhaging, lesions or edema, either externally or on any internal organs upon euthanasia and subsequent surgery. Similarly, there were no notable changes in behavior observed during the period of infection. This is likely due to the relatively short infection period utilized in this experiment. As noted in the introduction, infected animals often take 8-10 days, or more, to become symptomatic upon infection.

Gene clustering and functional annotation

We identified 263 probe sets with statistically significant differences in mRNA abundances between Day 0 and any other subsequent time point (Tables 1, 2). We assume that statistically significant probe sets correspond to genes that were differentially regulated after ATV infection. Cluster analysis of the statistically significant genes identified two groups that exhibited similar changes in mRNA abundance. After ATV infection at Day 0, 158 putative genes showed a significant increase in mRNA abundance at subsequent time points (Figure 2), while 105 transcripts showed a significant decrease (Figure 3). Thus, more genes were up-

regulated than down-regulated in response to ATV infection. Overall, DAVID categorized statistically significant genes among 44 different groups that correspond to different biological processes. Eight of these groups contained more genes than would be expected by chance sampling of genes from the microarray (geometric mean *P*-value < 0.05); these groups were considered significantly enriched with candidate genes relative to other groups (Table 3). Four of these significant groups contain gene ontologies related to immune response and pathogen response, including innate immunity, complement activation, lysosome function, and antigen processing and presentation. The most enriched functional group contains genes primarily related to immune function and defense responses. The remaining four functional groups contain gene ontologies related to ion binding, ion transport, vitamin metabolism, and response to an unfolded protein. Many genes that were classified in broader biological process categories that are not directly immunity-related are nonetheless associated with immunity in vertebrates (e.g. Hefti et al. 1999, van der Laan et al. 1997, Grassadonia et al. 2004).

Genes Up-regulated in Response to ATV

Across all time points, the majority of up-regulated genes were related to immune response or other related functions, such as inflammation and apoptosis. Other up-regulated genes pertained to gene functions such as ion binding and transport, membrane related functions, and protein binding and modification. Twenty-three genes (represented by 26 probe sets) demonstrated 2-fold or greater changes at 24 hours post-infection, all of which were up-regulated. Ten of these 23 have functions pertaining to immune response. Of the remaining highly expressed genes, one was associated with inflammation, two to regulation of apoptosis, three to ion binding, three to protein binding and modification, one to transport, one to the

extracellular constituent, and one to membrane and glycolipids. Many of these genes showed increasing transcript abundances over time. At 72 hours post infection, 43 genes had a greater than 5-fold change, and 40 genes had a greater than 5-fold change at 144 hours. The highest expression level, 91-fold increase at 144 hours, was observed for *interferon-induced protein with tetracopeptide repeats 5* (IFIT5).

Genes Down-regulated in Response to ATV

In contrast to the very high fold changes observed among up-regulated genes, the largest fold change observed among down-regulated genes was approximately 4.9-fold, in *chondroitin sulfate proteoglycan* (NCAN). Five down-regulated genes each code for regulation of transcription and translation. An additional 15 down-regulated genes correspond to 20 probe sets that have functions associated with cell division and mitosis, which was not observed in the up-regulated genes. Other notable down-regulated gene ontologies include one gene corresponding to pinocytosis and endocytosis, and one gene related to natural killer cell mediated cytotoxicity.

Validation of Microarray Results Using Quantitative Real-time PCR

We used qPCR to estimate fold changes for nine genes to verify our microarray data (Table 4). For five of the nine genes investigated (56%; *Myxovirus resistance 1, Macrophage receptor with collagenous structure, Complement component 3, Cyclin dependant kinase inhibitor 1B, Vaccinia related kinase 1*) there is good agreement between the microarray and qPCR data. In genes where the microarray estimates of fold change were modest (*Serine dehydratase like , Hemoglobin gamma alpha, Glycogen synthase kinase, Programmed cell death*

8) there is poorer agreement between fold change estimates from these two technologies. However, for this latter group of genes with modest fold change values, the microarray and qPCR data were always within four fold of each other. These results demonstrate that we were able to verify robust differences that were suggested by the microarray data.

Analyses to Identify Proliferation Gene Expression Signatures

Comparison of gene expression after ATV and tail amputation identified 25 genes that are significantly up-regulated in both experimental frameworks (Table 5). No significantly down-regulated genes were identified in common. Several of the commonly up-regulated genes appear to be related to humoral immunity, and membrane and extracellular matrix related functions. Additionally, general stress response genes such as *heat shock 70kDa protein 5* were similarly regulated. None of the cell cycle genes that are significantly up-regulated during tail regeneration were identified in this study. Thus, there was no evidence of cell proliferation by spleen cells after ATV infection.

Discussion

Emerging infectious diseases are implicated in the global decline of amphibians and other animals (Daszak et al. 2000, Stuart et al. 2004, Daszak et al. 2003, Williams et al. 2002). There is urgent need to develop understanding of amphibian immunological responses to pathogens and to identify host genes that may be important in disease resistance. Our study shows that functional genomics provides a means to rapidly meet these needs. We infected Mexican axolotls from the Ambystoma Genetic Stock Center with a viral pathogen that is clearly affecting tiger salamander populations in nature (Collins et al. 1988, Bollinger et al. 1999, Jancovich et al.

1997, Jancovich et al. 2005, Storfer et al. 2007). Our results show that ATV infection induces transcriptional changes of genes that are known to function in vertebrate immunity. Below we discuss the transcriptional response in more detail and suggest hypotheses to explain why ATV is often lethal to axolotls and other tiger salamanders.

We detected significant gene expression changes 24 hours post infection. Many of these gene expression changes likely reflect transcription within lymphocytes, as they are the predominant cell type in the spleen of juvenile and adult axolotls (Charlemagne and Tournefier 1998). Indeed, the functions of many of these genes are associated with neutrophil, dendritic, and macrophage cell functions, including cytokine signaling (*chemokine (C-X-C motif) receptor 4*), phagocytosis and destruction of phagocytised particles (*disabled homolog 2, mitogen-responsive phosphoprotein, neutrophil cytosolic factor 2*, *lysosomal-associated membrane protein 1*, *RAS homolog gene family, member B*), complement (*complement factor B*, *complement component 3*), and inflammation (*pentraxin related gene, rapidly induced by IL-1 beta*, *cytochrome B-245 beta polypeptide*, *n-myc and STAT interactor*). Up-regulation of complement components that are known to function in the removal of viral particles, and up-regulation of the stress-associated transcription factor *jun-b*, clearly shows that ATV induced an innate gene expression response in the axolotl. Further support for this idea was obtained by comparing ATV-induced gene expression changes to changes identified from a previous microarray experiment using *A. mexicanum* and the same microarray platform. Twenty-five genes that were up-regulated in response to ATV infection were also identified as significantly up-regulated during regeneration (Monaghan et al. 2007). In both microarray studies, blood was not perfused from tissues prior to tissue collection and it is known that leukocyctes express genes during the early wound-healing phase of spinal cord and limb regeneration. Thus, it seems likely that many of the early gene

expression changes that we observed in response to ATV-infection reflect a general, humoral transcriptional response to stress.

In addition to this general humoral response, the gene expression patterns that we observed suggest that the Mexican axolotl manifests an antiviral transcriptional response that is not unlike that observed in other vertebrates. For example, ATV infection clearly induces an interferon-mediated, antiviral response. Although probe sets for interferon genes are not represented on the GeneChip, we estimate based upon literature surveys that at least 20% of the significant genes that we identified are known in other systems (*in vitro* and *in vivo*) to be involved in interferon-mediated transcription (Hossain et al. 2006, Manger and Relman 2000, Jenner and Young 2005). These genes exhibited some of the largest fold-changes and include two primary transcription factors that compete to activate (*interferon regulatory factor 1*, up-regulated) and repress (*interferon regulatory factor 2*, down-regulated) transcription of interferon-alpha and beta (Type 1 interferon), and inferon-inducible genes that recognize and degrade intra-cellular viral nucleic acid (*interferon induced with helicase C domain 1*). Considering further that four of the most highly enriched functional groups also contained genes relating to the immune response and pathogen response, the results show that axolotls mount an anti-viral response that is greater than expected by random chance from 24-144 hours post-infection.

Given the robust immunological transcription response that we observed, it is curious why ATV is so virulent to tiger salamanders. In the closely related *Ranavirus* frog virus 3 (FV3), larval *Xenopus laevis* succomb to FV3 but adults effectively clear virons and develop lasting resistance to future infection (Maniero et al. 2006). Adult resistance in *X. laevis* is correlated with a significant proliferation of cytotoxic $CD8^+$ T cells in the spleen upon infection (within 6

days), as well as increased mortality upon $CD8^+$ T cell depletion (Morales and Robert 2007, Robert et al. 2005). Mortality events due to ATV are more significant among larvae in natural tiger salamander populations, however metamorphosed adult tiger salamanders are more susceptible than larvae to ATV infection in the lab (Brunner et al. 2005). It is well established that Mexican axolotls have a less complicated immune system and never develop the type of mature immune response typical of amniote vertebrates (Kaufman et al. 1995, Tournefier et al. 1998, Fellah and Charlemagne 1998, Tournefier et al. 1998, Charlemagne and Tournefier 1977, Kaufman et al. 1990, Koniski and Cohen 1992, Charlemagne 1979, Charlemagne 1991, Charlemagne and Tournefier 1998). We did not observe any gene expression changes that would indicate proliferative leukocyte responses in axolotl spleen. Perhaps this is because we used juvenile axolotls that are incapable of such a response. However, it is also possible that ATV maybe more resistant to the immune response mounted by *A. mexicanum* than FV3 is to the *Xenopus* immune response. Phylogenetic analyses indicate ATV is more closely related to iridoviruses found in fish than to FV3, which suggests a relatively recent host switch occurring with the introduction of sportfish to areas of the southwestern United States (Jancovich et al. 2005). Iridoviruses found in sportfish have a larger genome and contain more ORFs related to immune evasion than FV3, which could also be related to improved performance of this virus on the salamander host (Jancovich et al. 2005). Further studies are needed to better understand the ontogeny of immunological responses in axolotls, the virulence of different ranaviruses, and the role of innate versus adaptive immunity in ATV infection.

Our study has identified hundreds of new candidate genes for laboratory and field studies of stress and disease in tiger salamanders. Significantly more gene candidates will undoubtedly be discovered using a higher content, 2^{nd} generation microarray that is currently under

development. Genomic and bioinformatics tools make *Ambystoma* a powerful system for wildlife disease research. In particular, molecular information can be quickly cross-referenced from a genetically homogeneous strain that is available for laboratory studies (Mexican axolotl), to other closely related tiger salamander species in North America (Putta et al. 2004). Such power is needed to quickly understand how ATV and other pathogens are overwhelming amphibian immune responses and causing population declines in nature.

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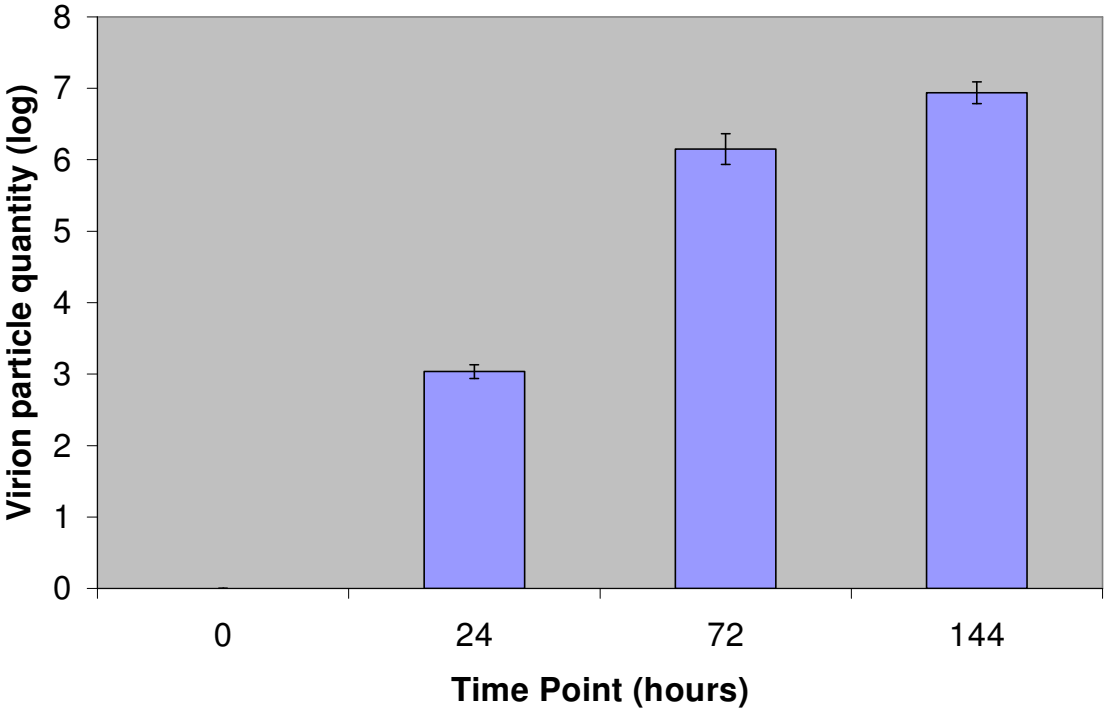
Figure 1. Log values of viral particles quantified with quantitative real-time PCR across all time points.



Figures 2 and 3. Expression profiles for clusters 1 and 2, respectively. Each row represents an individual gene, and each column a post-infection time point. Darker red coloration indicates increased expression of a gene relative to uninfected animals, and darker green indicates decreased expression.

Figure 2: Genes (Cluster 1, n=158) significantly up-regulated in response to ATV infection.

Fig 2 (cont'd)

MANNOSE-6-PHOSPHATE RECEPTOR BINDING PROTEIN 1
UDP-N-ACTEYLGLUCOSAMINE PYROPHOSPHORYLASE 1
tripartite motif-containing 17
MACROPHAGE EXPRESSED GENE 1
"INHIBITOR OF DNA BINDING 3, DOMINANT NEGATIVE HELIX-LOOP-HELIX PROTEIN"
"FER-1-LIKE 3, MYOFERLIN (C. ELEGANS)"
ELASTIN MICROFIBRIL INTERFACER 1
fibulin 1
"SOLUTE CARRIER FAMILY 15, MEMBER 3"
CATHEPSIN K (PYCNODYSOSTOSIS)
CD63 ANTIGEN (MELANOMA 1 ANTIGEN)
"CYTOCHROME B-245, BETA POLYPEPTIDE (CHRONIC GRANULOMATOUS DISEASE)"
CYTOCHROME C OXIDASE SUBUNIT VIB POLYPEPTIDE 1 (UBIQUITOUS)
"PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, BETA TYPE, 9 (LARGE MULTIFUNCTIONAL PEPTIDASE 2)"
"pyruvate kinase, muscle"
INTERFERON INDUCED TRANSMEMBRANE PROTEIN 5
HYPOTHETICAL PROTEIN MGC16212
tripartite motif-containing 39
NIDOGEN 2 (OSTEONIDOGEN)
MYELOPEROXIDASE
SYNDECAN BINDING PROTEIN (SYNTENIN)
LYSYL OXIDASE-LIKE 1
"CASPASE RECRUITMENT DOMAIN FAMILY, MEMBER 6"
"CYTOCHROME B-245, BETA POLYPEPTIDE (CHRONIC GRANULOMATOUS DISEASE)"
HEAT SHOCK 70KDA PROTEIN 8
PROTECTIVE PROTEIN FOR BETA-GALACTOSIDASE (GALACTOSIALIDOSIS)
GLYCINE N-METHYLTRANSFERASE
"DNAJ (HSP40) HOMOLOG, SUBFAMILY B, MEMBER 1"
CHROMOSOME 17 OPEN READING FRAME 27
THREE PRIME REPAIR EXONUCLEASE 2
"SOLUTE CARRIER FAMILY 11 (PROTON-COUPLED DIVALENT METAL ION TRANSPORTERS), MEMBER 1"
"SERPIN PEPTIDASE INHIBITOR, CLADE H (HEAT SHOCK PROTEIN 47), MEMBER 1, (COLLAGEN BINDING PROTEIN 1)"
"FRUCTOSE-1,6-BISPHOSPHATASE 1"
RELATED RAS VIRAL (R-RAS) ONCOGENE HOMOLOG 2
"pyruvate kinase, muscle"
"UDP-GLCNAC:BETAGAL BETA-1,3-N-ACETYLGLUCOSAMINYLTRANSFERASE 5"
"HAIRY AND ENHANCER OF SPLIT 1, (DROSOPHILA)"
"ARGINASE, TYPE II"
"GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE, GAMMA"
CATHEPSIN S
POLO-LIKE KINASE 3 (DROSOPHILA)
PRE-B-CELL COLONY ENHANCING FACTOR 1
CHEMOKINE (C-X-C MOTIF) RECEPTOR 4
JUN B PROTO-ONCOGENE
CLUSTERIN
MACROPHAGE RECEPTOR WITH COLLAGENOUS STRUCTURE
TISSUE FACTOR PATHWAY INHIBITOR 2
"GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE, BETA"
ARRESTIN DOMAIN CONTAINING 2
RETINOIC ACID RECEPTOR RESPONDER (TAZAROTENE INDUCED) 1
V-SET AND IMMUNOGLOBULIN DOMAIN CONTAINING 4
"MATRIX METALLOPEPTIDASE 9 (GELATINASE B, 92KDA GELATINASE, 92KDA TYPE IV COLLAGENASE)"
"HEAT SHOCK 70KDA PROTEIN 5 (GLUCOSE-REGULATED PROTEIN, 78KDA)"
INTERFERON INDUCED TRANSMEMBRANE PROTEIN 3 (1-8U)
"2'-5'-OLIGOADENYLATE SYNTHETASE 3, 100KDA"
ADIPOSE DIFFERENTIATION-RELATED PROTEIN
INTERFERON REGULATORY FACTOR 1
COMPLEMENT COMPONENT 3
"RIBONUCLEASE L (2',5'-OLIGOISOADENYLATE SYNTHETASE-DEPENDENT)"
INTERFERON-INDUCED PROTEIN 44
MATRIX METALLOPEPTIDASE 1 (INTERSTITIAL COLLAGENASE)
"TRANSCOBALAMIN I (VITAMIN B12 BINDING PROTEIN, R BINDER FAMILY)"
INTERFERON REGULATORY FACTOR 1
GASTRIC INTRINSIC FACTOR (VITAMIN B SYNTHESIS)
"TRANSGLUTAMINASE 1 (K POLYPEPTIDE EPIDERMAL TYPE I, PROTEIN-GLUTAMINE-GAMMA-GLUTAMYLTRANSFERASE)"
"PENTRAXIN-RELATED GENE, RAPIDLY INDUCED BY IL-1 BETA"
TIMP METALLOPEPTIDASE INHIBITOR 1
COMPLEMENT FACTOR B
"LECTIN, GALACTOSIDE-BINDING, SOLUBLE, 3 BINDING PROTEIN"
"LECTIN, GALACTOSIDE-BINDING, SOLUBLE, 3 BINDING PROTEIN"
"MYXOVIRUS (INFLUENZA VIRUS) RESISTANCE 1, INTERFERON-INDUCIBLE PROTEIN P78 (MOUSE)"
"MYXOVIRUS (INFLUENZA VIRUS) RESISTANCE 1, INTERFERON-INDUCIBLE PROTEIN P78 (MOUSE)"
INTERFERON INDUCED WITH HELICASE C DOMAIN 1
INTERFERON INDUCED WITH HELICASE C DOMAIN 1
INTERFERON-INDUCED PROTEIN WITH TETRATRICOPEPTIDE REPEATS 5

Figure 3: Genes (Cluster 2, n=105) significantly down-regulated in response to ATV infection.

Figure 3 (cont'd)

Table 1. Genes that were significantly up-regulated at any time point. Numbers indicate fold change at that time point.

Gene ID	Gene Name	24 hr	72 hr	144 hr
immune response				
SRV_03329_at	INTERFERON-INDUCED PROTEIN WITH TETRATRICOPEPTIDE REPEATS 5	11.85	82.22	91.04
SRV_01342_at	INTERFERON REGULATORY FACTOR 1	1.94	12.71	12.79
SRV_01343_a_at	INTERFERON REGULATORY FACTOR 1	2.10	16.07	15.63
SRV_13637_a_at	INTERFERON INDUCED TRANSMEMBRANE PROTEIN 3 (1-8U)	1.75	8.48	10.88
SRV_01199_a_at	CLUSTERIN	1.05	4.35	8.02
SRV_00442_at	SOLUTE CARRIER FAMILY 11 (PROTON-COUPLED DIVALENT METAL ION TRANSPORTERS), MEMBER 1	1.28	6.17	5.38
SRV_01303_a_at	GUANYLATE BINDING PROTEIN 1, INTERFERON-INDUCIBLE, 67KDA	1.10	2.35	2.49
SRV_02828_at	2'-5'-OLIGOADENYLATE SYNTHETASE 3, 100KDA	2.47	13.56	9.35
SRV_02072_at	CATHEPSIN S	1.54	5.51	6.43
SRV_02588_a_at	LECTIN, GALACTOSIDE-BINDING, SOLUBLE, 3 BINDING PROTEIN	4.04	26.37	24.40
SRV_02586_at	LECTIN, GALACTOSIDE-BINDING, SOLUBLE, 3 BINDING PROTEIN	3.88	21.30	18.03
response to virus				
SRV_03073_at	INTERFERON-INDUCED PROTEIN 44	2.05	9.44	13.16
SRV_01439_a_at	MYXOVIRUS (INFLUENZA VIRUS) RESISTANCE 1, INTERFERON-INDUCIBLE PROTEIN P78 (MOUSE)	3.13	29.23	36.80
SRV_01441_at	MYXOVIRUS (INFLUENZA VIRUS) RESISTANCE 1, INTERFERON-INDUCIBLE PROTEIN P78 (MOUSE)	3.26	28.24	35.84
SRV_04604_s_at	INTERFERON INDUCED WITH HELICASE C DOMAIN 1	6.81	27.34	24.15
SRV_04604_at	INTERFERON INDUCED WITH HELICASE C DOMAIN 1	7.27	25.43	22.62
SRV_04518_at	RIBONUCLEASE L (2',5'-OLIGOISOADENYLATE SYNTHETASE-DEPENDENT)	3.30	7.38	8.66
cell defense response				
SRV_00353_at	NEUTROPHIL CYTOSOLIC FACTOR 2 (65KDA, CHRONIC GRANULOMATOUS DISEASE, AUTOSOMAL 2)	1.05	2.07	1.73
SRV_00264_a_at	MYELOPEROXIDASE	1.60	7.60	4.10
antigen processing and presentation				
SRV_05347_at	PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, BETA TYPE, 9 (LARGE MULTIFUNCTIONAL PEPTIDASE 2)	1.27	3.73	4.25
lysosome/ endosome				
SRV_02581_a_at	LYSOSOMAL-ASSOCIATED MEMBRANE PROTEIN 1	1.23	2.26	2.06
SRV_12596_at	EH-DOMAIN CONTAINING 1	1.22	4.16	3.47
inflammatory response				
SRV_10702_at	CYTOCHROME B-245, BETA POLYPEPTIDE (CHRONIC GRANULOMATOUS DISEASE)	1.69	5.58	4.40
SRV_00330_at	CYTOCHROME B-245, BETA POLYPEPTIDE (CHRONIC GRANULOMATOUS DISEASE)	1.59	4.74	3.89
SRV_01877_at	CHEMOKINE (C-X-C MOTIF) RECEPTOR 4	1.29	5.70	8.08
SRV_02292_at	N-myc (and STAT) interactor	1.15	2.29	2.29
SRV_00453_a_at	TRANSFORMING GROWTH FACTOR, BETA 1 (CAMURATI-ENGELMANN DISEASE)	1.12	2.19	2.18
SRV_01617_a_at	PENTRAXIN-RELATED GENE, RAPIDLY INDUCED BY IL-1 BETA	2.83	12.82	17.76
cell cycle				
SRV_02067_at	POLO-LIKE KINASE 3 (DROSOPHILA)	0.93	3.82	6.18
SRV_11882_s_at	RAS HOMOLOG GENE FAMILY, MEMBER B	1.18	2.33	2.40
SRV_02051_at	RAS HOMOLOG GENE FAMILY, MEMBER B	1.26	2.15	2.28
SRV_02050_at	RAS HOMOLOG GENE FAMILY, MEMBER B	1.23	2.51	2.55

SRV_00154_at	EXOSTOSES (MULTIPLE) 1	0.90	2.07	1.90
glycolysis				
SRV_01520_at	pyruvate kinase, muscle	1.41	4.60	4.22
SRV_01519_a_at	pyruvate kinase, muscle	1.56	5.94	5.48
B_s_at	ALDOLASE A, FRUCTOSE-BISPHOSPHATE	1.22	3.14	2.96
transcription				
SRV_01351_at	JUN B PROTO-ONCOGENE	1.76	7.82	7.54
SRV_01336_a_at	INHIBITOR OF DNA BINDING 3, DOMINANT NEGATIVE HELIX-LOOP-HELIX PROTEIN	1.56	3.14	3.57
SRV_02310_at	EUKARYOTIC TRANSLATION TERMINATION FACTOR 1	0.95	2.70	2.45
SRV_03646_at	SPEN HOMOLOG, TRANSCRIPTIONAL REGULATOR (DROSOPHILA)	1.15	2.22	2.30
SRV_02571_at	HAIRY AND ENHANCER OF SPLIT 1, (DROSOPHILA)	1.23	4.52	6.10
complement				
SRV_00137_a_at	COMPLEMENT COMPONENT 3	1.80	9.79	13.66
SRV_01145_a_at	COMPLEMENT FACTOR B	3.46	18.33	20.05
apoptosis				
SRV_02399_a_at	MATRIX METALLOPEPTIDASE 9 (GELATINASE B, 92KDA GELATINASE, 92KDA TYPE IV COLLAGENASE)	2.19	6.79	8.78
SRV_02516_at	HEAT SHOCK 70KDA PROTEIN 5 (GLUCOSE-REGULATED PROTEIN, 78KDA)	2.70	10.28	7.60
SRV_04970_a_at	CASPASE RECRUITMENT DOMAIN FAMILY, MEMBER 6	1.35	4.78	4.80
SRV_08154_a_at	HEAT SHOCK 70KDA PROTEIN 9B (MORTALIN-2)	1.18	3.45	2.71
SRV_04300_a_at	CYTOCHROME C, SOMATIC	1.30	2.80	2.64
SRV_02132_at	EUKARYOTIC TRANSLATION ELONGATION FACTOR 1 EPSILON 1	1.21	3.56	3.38
SRV_01812_a_at	HEAT SHOCK PROTEIN 90KDA BETA (GRP94), MEMBER 1	1.31	3.53	3.26
SRV_03753_at	GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE, BETA	1.39	7.34	8.89
SRV_03023_a_at	GROWTH ARREST AND DNA-DAMAGE-INDUCIBLE, GAMMA	1.00	3.73	5.99
metabolic process				
SRV_05147_a_at	SYNDECAN BINDING PROTEIN (SYNTENIN)	1.27	4.78	4.42
SRV_00332_a_at	glucose-6-phosphate dehydrogenase	1.32	4.55	3.35
SRV_00292_a_at	PHOSPHOMANNOMUTASE 2	1.14	2.61	2.65
SRV_01737_a_at	UDP-N-ACTEYLGLUCOSAMINE PYROPHOSPHORYLASE 1	1.84	2.18	2.59
SRV_05108_a_at	PHOSPHOSERINE AMINOTRANSFERASE 1	1.12	1.97	2.48
C_s_at	ALDOLASE A, FRUCTOSE-BISPHOSPHATE	1.15	2.42	2.27
A_s_at	ALDOLASE A, FRUCTOSE-BISPHOSPHATE	1.24	3.64	3.39
SRV_00129_a_at	ALDOLASE B, FRUCTOSE-BISPHOSPHATE	1.26	2.30	2.13
SRV_02002_at	CARBOXYLESTERASE 1 (MONOCYTE/MACROPHAGE SERINE ESTERASE 1)	1.28	3.86	2.38
signal transduction				
SRV_02657_at	PRE-B-CELL COLONY ENHANCING FACTOR 1	1.61	7.61	7.01
SRV_03054_at	MACROPHAGE RECEPTOR WITH COLLAGENOUS STRUCTURE	2.17	8.20	6.82
SRV_01818_at	THIOREDOXIN	1.19	3.29	3.51
SRV_01313_a_at	GRANULIN	1.45	3.72	3.40
SRV_01462_at	NUCLEAR FACTOR OF KAPPA LIGHT POLYPEPTIDE GENE ENHANCER IN B-CELLS 2 (P49/P100)	1.59	3.67	2.77
SRV_03006_a_at	IQ MOTIF CONTAINING GTPASE ACTIVATING PROTEIN 2	1.23	2.59	2.69
SRV_01480_at	PURINERGIC RECEPTOR P2Y, G-PROTEIN COUPLED, 2	1.47	3.17	2.44
SRV_02894_a_at	CAP, ADENYLATE CYCLASE-ASSOCIATED PROTEIN 1 (YEAST)	1.10	2.09	1.76
SRV_00844_a_at	CHLORIDE INTRACELLULAR CHANNEL 1	1.15	1.95	2.10
membrane				
SRV_03227_at	NIDOGEN 2 (OSTEONIDOGEN)	1.42	3.69	4.29
SRV_01179_a_at	CD63 ANTIGEN (MELANOMA 1 ANTIGEN)	1.34	3.82	3.97
SRV_02687_s_at	MANNOSE-6-PHOSPHATE RECEPTOR BINDING PROTEIN 1	1.30	4.82	3.55
SRV_04888_a_at	UDP-GLCNAC:BETAGAL BETA-1,3-N-	2.41	4.44	3.50

	ACETYLGLUCOSAMINYLTRANSFERASE 5			
SRV_04819_a_at	TRANSMEMBRANE PROTEIN 49	1.46	3.40	3.20
SRV_04070_a_at	LEUCINE RICH REPEAT CONTAINING 59	1.22	2.48	2.78
SRV_03407_at	FER-1-LIKE 3, MYOFERLIN (C. ELEGANS)	1.22	2.67	3.81
SRV_05439_at	HYPOTHETICAL PROTEIN LOC441168	1.40	3.43	2.79
SRV_02874_a_at	BRAIN ABUNDANT, MEMBRANE ATTACHED SIGNAL PROTEIN 1	1.33	2.80	2.31
SRV_04226_a_at	GTPASE, IMAP FAMILY MEMBER 5	1.13	2.05	2.02
transport				
SRV_00744_a_at	ADIPOSE DIFFERENTIATION-RELATED PROTEIN	3.49	12.49	7.34
SRV_00294_s_at	PROTECTIVE PROTEIN FOR BETA-GALACTOSIDASE (GALACTOSIALIDOSIS)	1.53	5.23	4.73
SRV_02592_a_at	LYSYL OXIDASE-LIKE 1	1.18	2.81	4.59
SRV_03991_a_at	SOLUTE CARRIER FAMILY 15, MEMBER 3	1.36	5.33	3.88
SRV_03562_at	ERO1-LIKE (S. CEREVISIAE)	1.00	2.24	1.77
SRV_04996_at	SOLUTE CARRIER FAMILY 7 (CATIONIC AMINO ACID TRANSPORTER, Y+ SYSTEM), MEMBER 3	1.07	1.75	2.11
SRV_01134_at	ATPASE, H+ TRANSPORTING, LYSOSOMAL 56/58KDA, V1 SUBUNIT B2	1.04	2.17	1.59
SRV_01220_at	CYTOCHROME C OXIDASE SUBUNIT VIB POLYPEPTIDE 1 (UBIQUITOUS)	1.15	4.51	4.18
SRV_02133_a_at	GOLGI SNAP RECEPTOR COMPLEX MEMBER 2	1.05	2.24	1.88
extracellular region				
SRV_02948_a_at	fibulin 1	0.93	1.65	2.24
SRV_01275_at	fibulin 1	0.81	1.54	2.10
SRV_02965_at	TISSUE FACTOR PATHWAY INHIBITOR 2	3.56	3.04	2.56
SRV_03142_at	ELASTIN MICROFIBRIL INTERFACER 1	1.11	3.37	3.73
ion binding				
SRV_00713_a_at	TRANSCOBALAMIN I (VITAMIN B12 BINDING PROTEIN, R BINDER FAMILY)	2.55	23.02	12.98
SRV_02456_at	GASTRIC INTRINSIC FACTOR (VITAMIN B SYNTHESIS)	3.36	20.67	11.63
SRV_07722_at	CHROMOSOME 17 OPEN READING FRAME 27	0.94	4.46	4.63
SRV_05460_at	tripartite motif-containing 39	1.58	3.81	4.07
SRV_05065_at	tripartite motif-containing 17	1.53	2.86	3.46
SRV_00371_a_at	FRUCTOSE-1,6-BISPHOSPHATASE 1	2.40	5.01	3.14
SRV_00741_a_at	ADENOSINE DEAMINASE, RNA-SPECIFIC	1.44	3.76	2.86
SRV_00562_a_at	PROSTAGLANDIN-ENDOPEROXIDE SYNTHASE 1 (PROSTAGLANDIN G/H SYNTHASE AND CYCLOOXYGENASE)	1.29	2.05	2.54
SRV_05448_at	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	0.87	2.16	2.13
SRV_02724_at	METHIONINE ADENOSYLTRANSFERASE II, ALPHA	1.66	2.06	1.56
SRV_00449_a_at	LATENT TRANSFORMING GROWTH FACTOR BETA BINDING PROTEIN 1	1.05	1.79	3.15
SRV_00131_a_at	SECRETED PROTEIN, ACIDIC, CYSTEINE-RICH (OSTEONECTIN)	1.19	1.99	2.74
SRV_04896_a_at	TRANSKETOLASE (WERNICKE-KORSAKOFF SYNDROME)	1.44	4.02	2.52
SRV_02047_at	ANNEXIN A2	1.29	2.15	2.45
SRV_01345_at	INOSITOL 1,4,5-TRIPHOSPHATE RECEPTOR, TYPE 3	1.19	2.80	2.35
SRV_12418_at	MYOSIN, LIGHT POLYPEPTIDE 9, REGULATORY	1.01	1.99	2.20
SRV_01649_at	RETICULOCALBIN 1, EF-HAND CALCIUM BINDING DOMAIN	1.16	3.01	2.68
protein binding/transport				
SRV_03206_at	V-SET AND IMMUNOGLOBULIN DOMAIN CONTAINING 4	1.90	13.14	9.17
SRV_00797_a_at	SERPIN PEPTIDASE INHIBITOR, CLADE H (HEAT SHOCK PROTEIN 47), MEMBER 1, (COLLAGEN BINDING PROTEIN 1)	1.77	4.67	4.79
SRV_04964_a_at	TUBULIN, BETA 6	1.06	2.53	2.55
SRV_03477_a_at	TRANSMEMBRANE PROTEIN 4	1.59	2.22	2.39
SRV_02085_at	FK506 BINDING PROTEIN 1B, 12.6 KDA	1.29	1.71	2.18
SRV_02652_a_at	PROTEIN DISULFIDE ISOMERASE FAMILY A, MEMBER 6	1.28	2.16	1.99
SRV_02814_at	DNAJ (HSP40) HOMOLOG, SUBFAMILY B, MEMBER 1	2.35	4.29	1.73
SRV_03255_a_at	AHA1, ACTIVATOR OF HEAT SHOCK 90KDA PROTEIN ATPASE HOMOLOG 1 (YEAST)	1.07	2.14	1.53
SRV_05534_at	HYPOTHETICAL PROTEIN MGC16212	1.74	4.05	3.73

SRV_00840_a_at	COLD INDUCIBLE RNA BINDING PROTEIN	1.03	2.46	3.20
SRV_01147_a_at	BIGLYCAN	0.99	2.01	2.90
SRV_05461_x_at	tripartite motif-containing 39	1.32	2.53	2.76
SRV_02070_a_at	CYSTEINE AND GLYCINE-RICH PROTEIN 1	1.27	2.22	2.46
SRV_00257_at	KERATIN 18	1.12	1.93	2.44
SRV_04005_at	KDEL (LYS-ASP-GLU-LEU) ENDOPLASMIC RETICULUM PROTEIN RETENTION RECEPTOR 3	0.90	2.07	2.06
SRV_02647_a_at	ARP2 ACTIN-RELATED PROTEIN 2 HOMOLOG (YEAST)	1.23	2.33	1.93
SRV_01234_a_at	CATENIN (CADHERIN-ASSOCIATED PROTEIN), ALPHA 1, 102KDA	0.98	1.43	2.05
SRV_01504_a_at	PHOSPHOGLUCONATE DEHYDROGENASE	1.19	2.20	1.50
SRV_05174_a_at	THREE PRIME REPAIR EXONUCLEASE 2	1.57	5.55	5.02
protein modification				
SRV_04305_a_at	GLYCINE N-METHYLTRANSFERASE	1.24	1.84	3.49
SRV_01832_a_at	UBIQUITIN-CONJUGATING ENZYME E2L 3	1.04	2.09	1.99
SRV_00309_at	TRANSGLUTAMINASE 1 (K POLYPEPTIDE EPIDERMAL TYPE I, PROTEIN-GLUTAMINE-GAMMA-GLUTAMYLTRANSFERASE)	3.22	16.60	14.36
SRV_02093_a_at	HEAT SHOCK 70KDA PROTEIN 9B (MORTALIN-2)	1.35	3.91	2.79
SRV_02989_at	HEAT SHOCK 70KDA PROTEIN 8	2.28	6.93	2.73
SRV_01225_at	CRYSTALLIN, ALPHA B	1.27	3.35	2.38
SRV_05456_a_at	UBIQUITIN SPECIFIC PEPTIDASE 2	1.27	2.56	2.00
SRV_05457_a_at	UBIQUITIN SPECIFIC PEPTIDASE 2	1.26	2.28	1.89
SRV_11417_a_at	MATRIX METALLOPEPTIDASE 1 (INTERSTITIAL COLLAGENASE)	5.26	13.96	3.65
SRV_04306_at	GLYCINE N-METHYLTRANSFERASE	1.03	2.15	4.66
SRV_00327_a_at	CATHEPSIN K (PYCNODYSOSTOSIS)	1.37	3.23	3.94
cellular process				
SRV_11663_a_at	TIMP METALLOPEPTIDASE INHIBITOR 1	1.96	24.74	23.31
SRV_04387_at	RETINOIC ACID RECEPTOR RESPONDER (TAZAROTENE INDUCED) 1	1.64	6.63	9.22
SRV_03285_at	RELATED RAS VIRAL (R-RAS) ONCOGENE HOMOLOG 2	1.76	5.07	4.97
SRV_04911_at	SIMILAR TO THIOREDOXIN DOMAIN-CONTAINING 2	1.34	3.15	3.20
SRV_01534_at	PROTEIN PHOSPHATASE 1, CATALYTIC SUBUNIT, ALPHA ISOFORM	1.15	2.04	2.18
SRV_04858_at	POLY (ADP-RIBOSE) POLYMERASE FAMILY, MEMBER 9	0.97	2.36	2.15
SRV_03421_a_at	LR8 PROTEIN	1.31	1.95	2.02
SRV_11406_at	V-YES-1 YAMAGUCHI SARCOMA VIRAL RELATED ONCOGENE HOMOLOG	1.37	2.46	2.12
other				
SRV_03887_at	DYNEIN, CYTOPLASMIC 1, LIGHT INTERMEDIATE CHAIN 1	1.31	2.42	2.20
SRV_01920_at	poly (ADP-ribose) glycohydrolase	1.31	1.95	2.23
SRV_00155_a_at	COAGULATION FACTOR XIII, A1 POLYPEPTIDE	1.20	2.46	2.35
SRV_01367_a_at	KERATIN 8	1.19	2.90	3.29
SRV_00775_at	ARGINASE, TYPE II	1.44	4.08	6.06
SRV_11767_a_at	INTERFERON INDUCED TRANSMEMBRANE PROTEIN 5	1.17	2.74	4.24
SRV_07726_a_at	MACROPHAGE EXPRESSED GENE 1	1.69	4.25	3.18
SRV_01302_at	GUANYLATE BINDING PROTEIN 4	1.07	2.39	2.64
SRV_02761_at	ARGININE-RICH, MUTATED IN EARLY STAGE TUMORS	1.10	2.17	1.99
SRV_03758_a_at	ARRESTIN DOMAIN CONTAINING 2	0.90	5.48	8.72

Table 2. Genes that were significantly down-regulated at any time point. Numbers indicate fold change at that time point.

ID	NAME	24 hr	72 hr	144 hr
transcription				
SRV_04230_at	CHROMOSOME X OPEN READING FRAME 15	0.73	0.44	0.50
SRV_01344_a_at	INTERFERON REGULATORY FACTOR 2	1.02	0.47	0.50
SRV_01768_a_at	TAF9 RNA POLYMERASE II, TATA BOX BINDING PROTEIN (TBP)-ASSOCIATED FACTOR, 32KDA	0.84	0.44	0.50
SRV_03843_a_at	MEDIATOR OF RNA POLYMERASE II TRANSCRIPTION, SUBUNIT 31 HOMOLOG (YEAST)	0.95	0.50	0.45
SRV_01892_at	ZINC FINGER PROTEIN 282	0.98	0.39	0.42
translation				
SRV_03800_a_at	MITOCHONDRIAL RIBOSOMAL PROTEIN S7	1.13	0.50	0.54
SRV_03598_at	MITOCHONDRIAL RIBOSOMAL PROTEIN L19	0.73	0.49	0.53
SRV_04607_at	PEPTIDE DEFORMYLASE-LIKE PROTEIN	1.01	0.46	0.50
SRV_04925_a_at	HYPOTHETICAL PROTEIN MGC11102	1.01	0.43	0.48
SRV_01958_at	EUKARYOTIC TRANSLATION INITIATION FACTOR 4E BINDING PROTEIN 3	1.05	0.32	0.31
Natural Killer cell mediated cytotoxicity				
AE_at	TUBULIN, BETA 2C	0.88	0.52	0.49
apoptosis				
SRV_11815_at	CASP2 AND RIPK1 DOMAIN CONTAINING ADAPTOR WITH DEATH DOMAIN	0.70	0.45	0.52
SRV_01489_at	PRKC, APOPTOSIS, WT1, REGULATOR	0.88	0.43	0.44
ion binding/ transport				
SRV_03020_at	TRAF-TYPE ZINC FINGER DOMAIN CONTAINING 1	0.85	0.49	0.53
SRV_01742_at	SPECTRIN, ALPHA, NON-ERYTHROCYTIC 1 (ALPHA-FODRIN)	0.82	0.46	0.52
SRV_02131_a_at	PEPTIDASE (MITOCHONDRIAL PROCESSING) BETA	0.80	0.48	0.51
SRV_02733_at	MITOCHONDRIAL INTERMEDIATE PEPTIDASE	0.59	0.39	0.40
SRV_04112_at	HYPOTHETICAL PROTEIN FLJ20699	0.93	0.42	0.39
SRV_00559_a_at	PRIMASE, POLYPEPTIDE 1, 49KDA	0.79	0.42	0.39
SRV_03126_at	RING FINGER PROTEIN 113A	0.95	0.39	0.39
SRV_03759_at	ATP SYNTHASE, H+ TRANSPORTING, MITOCHONDRIAL F0 COMPLEX, SUBUNIT S (FACTOR B)	0.91	0.30	0.25
SRV_01177_a_at	ECTONUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASE 1	1.19	0.52	0.49
SRV_04638_a_at	MEMBRANE-ASSOCIATED RING FINGER (C3HC4) 7	0.78	0.44	0.47
SRV_03403_at	MAKORIN, RING FINGER PROTEIN, 1	1.02	0.55	0.48
SRV_02137_at	GUANINE DEAMINASE	0.93	0.36	0.36
SRV_12156_at	TUMOR PROTEIN D52	0.93	0.58	0.45
SRV_02173_at	CHONDROITIN SULFATE PROTEOGLYCAN 3 (NEUROCAN)	1.38	0.57	0.20
metabolic process				
SRV_02346_a_at	GLUTATHIONE S-TRANSFERASE OMEGA 1	1.04	0.54	0.47
SRV_04215_at	PHOSPHOGLUCOMUTASE 2	1.08	0.57	0.46
SRV_04397_at	NITRILASE FAMILY, MEMBER 2	0.86	0.49	0.46
SRV_03369_at	NON-METASTATIC CELLS 7, PROTEIN EXPRESSED IN (NUCLEOSIDE-DIPHOSPHATE KINASE)	1.00	0.55	0.43
SRV_00123_at	AMINOLEVULINATE, DELTA-, DEHYDRATASE	0.71	0.49	0.31
SRV_00160_s_at	FUMARYLACETOACETATE HYDROLASE (FUMARYLACETOACETASE)	0.83	0.61	0.49
SRV_00135_at	ASPARTOACYLASE (CANAVAN DISEASE)	0.92	0.30	0.22
SRV_01499_at	6-PHOSPHOFRUCTO-2-KINASE/FRUCTOSE-2,6-BIPHOSPHATASE 1	0.88	0.65	0.47
SRV_11745_at	ACYL-COENZYME A OXIDASE 3, PRISTANOYL	0.87	0.51	0.47
SRV_03094_at	LIPOIC ACID SYNTHETASE	0.83	0.47	0.47
SRV_05217_a_at	SERINE DEHYDRATASE-LIKE	1.03	0.52	0.33

transport				
SRV_03906_at	HEMATOPOIETIC STEM/PROGENITOR CELLS 176	0.81	0.49	0.58
SRV_04743_a_at	HYPOTHETICAL PROTEIN FLJ22028	0.98	0.54	0.50
SRV_02065_a_at	ADAPTOR-RELATED PROTEIN COMPLEX 2, MU 1 SUBUNIT	0.90	0.59	0.48
SRV_03218_a_at	GABA(A) RECEPTOR-ASSOCIATED PROTEIN-LIKE 2	0.93	0.28	0.37
SRV_05300_a_at	SFT2 DOMAIN CONTAINING 2	0.91	0.31	0.29
SRV_05537_a_at	TRAFFICKING PROTEIN PARTICLE COMPLEX 6B	0.95	0.49	0.34
SRV_02033_a_at	SELENIUM BINDING PROTEIN 1	1.05	0.58	0.45
protein binding/modification				
SRV_04235_at	hypothetical protein FLJ11280	0.68	0.44	0.56
SRV_02678_a_at	M-PHASE PHOSPHOPROTEIN 6	0.84	0.45	0.51
SRV_02198_a_at	GLUTAMYL-PROLYL-TRNA SYNTHETASE	0.94	0.52	0.48
SRV_01481_at	PHOSPHATIDYLETHANOLAMINE BINDING PROTEIN 1	1.05	0.56	0.49
SRV_01495_at	PYRUVATE DEHYDROGENASE KINASE, ISOZYME 2	1.04	0.41	0.31
SRV_04077_a_at	UBIQUITIN-CONJUGATING ENZYME E2R 2	0.78	0.51	0.47
SRV_04977_s_at	CNDP DIPEPTIDASE 2 (METALLOPEPTIDASE M20 FAMILY)	1.27	0.49	0.22
SRV_04977_at	CNDP DIPEPTIDASE 2 (METALLOPEPTIDASE M20 FAMILY)	1.20	0.46	0.22
SRV_01825_at	UBIQUITIN-CONJUGATING ENZYME E2B (RAD6 HOMOLOG)	0.90	0.47	0.51
RNA binding/processing				
SRV_03823_at	RNA BINDING MOTIF PROTEIN, X-LINKED 2	0.80	0.47	0.54
SRV_03721_at	SYF2 HOMOLOG, RNA SPLICING FACTOR (S. CEREVISIAE)	0.96	0.46	0.49
SRV_03417_at	MITOCHONDRIAL RIBOSOMAL PROTEIN S28	0.86	0.51	0.47
SRV_03836_at	EXOSOME COMPONENT 1	1.03	0.46	0.46
cell cycle/ cell division				
SRV_05218_a_at	COILED-COIL DOMAIN CONTAINING 5 (SPINDLE ASSOCIATED)	0.68	0.36	0.46
SRV_03244_a_at	FREQUENTLY REARRANGED IN ADVANCED T-CELL LYMPHOMAS 2	0.89	0.38	0.46
SRV_05024_at	ZW10 INTERACTOR	0.96	0.41	0.44
SRV_00804_at	CDC6 CELL DIVISION CYCLE 6 HOMOLOG (S. CEREVISIAE)	0.70	0.50	0.42
SRV_03256_at	TPX2, MICROTUBULE-ASSOCIATED, HOMOLOG (XENOPUS LAEVIS)	0.91	0.41	0.51
SRV_04156_at	CELL DIVISION CYCLE ASSOCIATED 8	0.71	0.43	0.50
SRV_03593_at	DISCS, LARGE HOMOLOG 7 (DROSOPHILA)	0.66	0.39	0.49
SRV_14350_at	NIMA (NEVER IN MITOSIS GENE A)-RELATED KINASE 3	0.96	0.42	0.30
SRV_02556_at	SMC4 STRUCTURAL MAINTENANCE OF CHROMOSOMES 4-LIKE 1 (YEAST)	0.69	0.43	0.47
SRV_03257_at	TPX2, MICROTUBULE-ASSOCIATED, HOMOLOG (XENOPUS LAEVIS)	0.79	0.35	0.45
SRV_02235_at	KINESIN FAMILY MEMBER 11	0.53	0.31	0.44
SRV_01290_at	FERRITIN, HEAVY POLYPEPTIDE 1	1.00	0.54	0.42
SRV_02151_a_at	CENTRIN, EF-HAND PROTEIN, 2	0.65	0.39	0.39
SRV_04253_a_at	NUCLEOLAR AND SPINDLE ASSOCIATED PROTEIN 1	0.62	0.28	0.37
SRV_05141_at	CYCLIN-DEPENDENT KINASE INHIBITOR 2C (P18, INHIBITS CDK4)	0.85	0.57	0.37
SRV_00033_copy4_at	T-cell acute lymphocytic leukemia 1	1.02	0.47	0.36
SRV_00033_at	T-cell acute lymphocytic leukemia 1	0.97	0.44	0.35
SRV_00033_copy2_at	T-cell acute lymphocytic leukemia 1	0.96	0.46	0.34
SRV_00033_copy1_at	T-cell acute lymphocytic leukemia 1	0.96	0.44	0.33
SRV_00033_copy3_at	T-cell acute lymphocytic leukemia 1	0.94	0.44	0.33
membrane				
SRV_04260_at	CHROMOSOME 9 OPEN READING FRAME 46	1.05	0.56	0.47
SRV_04763_at	CHROMOSOME 10 OPEN READING FRAME 57	1.24	0.46	0.42
SRV_04650_a_at	TRANSMEMBRANE 6 SUPERFAMILY MEMBER 1	1.00	0.47	0.32
SRV_05571_a_at	OXIDATION RESISTANCE 1	0.77	0.37	0.42
SRV_03611_a_at	TRANSLOCASE OF OUTER MITOCHONDRIAL	0.97	0.55	0.48

	MEMBRANE 70 HOMOLOG A (YEAST)			
kinase activity				
SRV_05333_at	RIO kinase 3 (yeast)	1.06	0.47	0.52
SRV_05450_a_at	INTEGRIN BETA 1 BINDING PROTEIN 3	0.87	0.53	0.46
SRV_01863_at	VACCINIA RELATED KINASE 1	0.61	0.39	0.51
pinocytosis/ endocytosis				
SRV_00866_at	DISABLED HOMOLOG 2, MITOGEN-RESPONSIVE PHOSPHOPROTEIN (DROSOPHILA)	0.84	0.49	0.43
DNA damage				
SRV_04199_at	NEI ENDONUCLEASE VIII-LIKE 3 (E. COLI)	0.75	0.41	0.37
SRV_02222_at	HUS1 CHECKPOINT HOMOLOG (S. POMBE)	0.72	0.45	0.57
muscle development/ contraction				
SRV_01033_a_at	INTERFERON-RELATED DEVELOPMENTAL REGULATOR 1	1.18	0.38	0.53
SRV_05143_a_at	MYOSIN, LIGHT POLYPEPTIDE 1, ALKALI; SKELETAL, FAST	0.79	0.50	0.49
SRV_00058_s_at	tropomyosin	0.89	0.53	0.48
other				
SRV_01932_a_at	FICOLIN (COLLAGEN/FIBRINOGEN DOMAIN CONTAINING) 3 (HAKATA ANTIGEN)	1.03	0.58	0.46
SRV_05356_s_at	FAMILY WITH SEQUENCE SIMILARITY 58, MEMBER A	0.89	0.46	0.47
SRV_02972_at	GLUTAREDOXIN 5 HOMOLOG (S. CEREVISIAE)	0.97	0.49	0.49
SRV_05356_at	FAMILY WITH SEQUENCE SIMILARITY 58, MEMBER A	1.09	0.58	0.58
SRV_05263_at	SOLUTE CARRIER FAMILY 39 (ZINC TRANSPORTER), MEMBER 3	0.73	0.45	0.51
SRV_04739_a_at	ZINC FINGER PROTEIN 403	0.97	0.49	0.51
SRV_03830_at	SHWACHMAN-BODIAN-DIAMOND SYNDROME	0.94	0.47	0.46
SRV_03448_at	COILED-COIL DOMAIN CONTAINING 59	0.89	0.42	0.44
SRV_02223_a_at	ISOPENTENYL-DIPHOSPHATE DELTA ISOMERASE 1	0.89	0.37	0.43
SRV_04160_at	SDA1 DOMAIN CONTAINING 1	0.88	0.48	0.42
SRV_05216_at	SERUM AMYLOID A-LIKE 1	0.82	0.42	0.42
SRV_04991_a_at	MYC INDUCED NUCLEAR ANTIGEN	0.90	0.41	0.39
SRV_00134_a_at	ARGININOSUCCINATE LYASE	1.10	0.44	0.36
SRV_05376_at	WILLIAMS BEUREN SYNDROME CHROMOSOME REGION 27	0.84	0.36	0.31

Table 3. Significant ($P \leq 0.05$ geometric mean p-value) functional groups obtained from functional annotation using DAVID.

	Ontology	Number of Genes	p-value			Ontology	Number of Genes	p-value
Functional Group 1	response to biotic stimulus	25	<0.001		Functional Group 5	water-soluble vitamin metabolism	4	0.02
<0.001	immune response	21	<0.001		0.033	vitamin metabolism	4	0.03
	defense response	22	<0.001			pyridine nucleotide metabolism	3	0.07
Functional Group 2	cation binding	34	0.001		Functional Group 6	di-, tri-valent inorganic cation transport	5	0.006
0.002	ion binding	36	0.004		0.042	metal ion transport	5	0.06
	metal ion binding	36	0.004			cation transport	6	0.22
Functional Group 3	innate immunity	4	0.001		Functional Group 7	bcr protein	3	0.02
0.005	immune response	5	0.004		0.045	molecular chaperone	4	0.02
	innate immune response	4	0.007			Heat shock protein Hsp70	3	0.03
	complement activation	3	0.02			Heat shock protein 70	3	0.03
						antigen processing and presentation	4	0.09
						cell surface	3	0.20
Functional Group 4	lysosome	7	0.002					
0.008	lysosome	7	0.009		Functional Group 8	response to unfolded protein	5	0.02
	lytic vacuole	7	0.009		0.048	response to protein stimulus	5	0.02
	vacuole	7	0.02			chaperone	6	0.26

Table 4. Fold changes obtained from microarray and from quantitative real-time PCR in order to verify microarray results.

Gene name	Microarray		qPCR	
	24	72	24	72
Myxovirus resistance 1 MX1	3.13	29.23	5.97	24.44
Macrophage receptor with collagenous structure MARCO	2.17	8.20	3.36	15.24
Complement component 3 C3	1.80	9.79	2.14	14.78
Cyclin dependant kinase inhibitor 1B CDKN1B	-1.17	-1.74	-2.26	-3.16
Vaccinia related kinase 1 VRK1	-1.64	-2.58	-1.23	-1.88
Serine dehydratase like SDSL	1.03	-1.92	-1.35	-1.01
Hemoglobin gamma alpha HBG1	-1.01	-1.02	-1.54	-1.87
Glycogen synthase kinase GSK3A	-1.07	-1.08	-1.13	2.76
Programmed cell death 8 PDCD8	1.13	-1.10	1.30	1.85

Table 5. Genes expressed in both ATV infection and spinal cord injury to conclude which genes are up-regulated in response to wounding, and which are in response to viral infection.

Gene ID	Gene Name	Gene Ontology
SRV_00294_s_at	protective protein for beta-galactosidase (galactosialidosis)	proteolysis, protein transport
SRV_00309_at	transglutaminase 1 (K polypeptide epidermal type I, protein-glutamine-gamma-glutamyltransferase)	membrane, cell envelope, protein modification
SRV_00327_a_at	cathepsin K (pycnodysostosis)	proteolysis
SRV_00330_at	cytochrome b-245, beta polypeptide (chronic granulomatous disease)	humoral response, inflammatory response
SRV_00371_a_at	fructose-1,6-bisphosphatase 1	metal ion binding (zinc)
SRV_00442_at	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	immune response, ion transport
SRV_00713_a_at	transcobalamin I (vitamin B12 binding protein, R binder family)	ion transport/binding (cobalt)
SRV_00744_a_at	adipose differentiation-related protein	fatty acid transport, extracellular region
SRV_01179_a_at	CD63 antigen (melanoma 1 antigen)	endosome, membrane
SRV_01342_at	interferon regulatory factor 1	immune response, transcription
SRV_01351_at	jun B proto-oncogene	transcription
SRV_01818_at	thioredoxin	signal transduction
SRV_02399_a_at	matrix metalloproteinase 9	extracellular matrix, apoptosis, proteolysis
SRV_02456_at	gastric intrinsic factor (vitamin B synthesis)	ion transport/binding (cobalt)
SRV_02516_at	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	anti-apoptosis, endoplasmic reticulum
SRV_02586_at	lectin, galactoside-binding, soluble, 3 binding protein	cell adhesion, cellular defense response, signal transduction
SRV_03054_at	macrophage receptor with collagenous structure	signal transduction
SRV_04604_at	interferon induced with helicase c domain 1	innate immune response, regulation of apoptosis, response to virus
SRV_04819_a_at	transmembrane protein 49	membrane, endoplasmic reticulum
SRV_04888_a_at	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	membrane, CNS development
SRV_04911_at	thioredoxin domain containing 2	cell redox homeostasis
SRV_04964_a_at	tubulin beta MGC4083	nucleotide binding, protein polymerization
SRV_07726_a_at	macrophage expressed gene 1	none
SRV_11417_a_at	matrix metalloproteinase 1 (interstitial collagenase)	proteolysis, ion binding (zinc)
SRV_11663_a_at	tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	enzyme inhibitor, cell proliferation
SRV_00294_s_at	protective protein for beta-galactosidase (galactosialidosis)	proteolysis, protein transport

A test of local adaptation in an emerging virus with spatial variation in selection

Abstract

Emerging infectious diseases impact both human and wildlife populations. In wildlife systems, these diseases impact both population stability and biodiversity. Disease emergence can occur when a pathogen enters new geographic range and infects hosts that do not share an evolutionary history. The outcome depends on the fitness of these pathogens in foreign hosts.

The amphibian pathogen *Ambystoma tigrinum* virus (ATV) has had a severe impact on salamander populations across the US. The virulence genes of some populations have been under strong positive selection, while selection has been weak or purifying in others. We hypothesized that positive selection is associated with pathogen local adaptation because these populations have not reached an adaptive peak. In contrast,, no selection on one or more genes should be associated with pathogen local adaptation. We estimated within-host viral growth and tested for pathogen local adaptation using four analyses: t-tests comparing sympatric versus allopatric strains on each host, a regression of viral growth rate against host genetic distance, home versus away analyses, and local versus foreign analyses.

We found partial support for the idea that positive selection on a virulence gene was associated with pathogen local adaptation. One population under positive selection was significantly locally maladapted, whereas the other was non-significantly locally adapted, or equivocal. With regards to the viruses with no or unknown selective pressures, they were both equivocal with the majority of estimates indicating a lack of local adaptation. Overall, there was no sign of local adaptation in this host-virus system.

This research not only examines whether a virus has become locally adapted to its host, but it also is a beginning in discovering how viruses coevolve with their vertebrate hosts. As the

vertebrate immune system imposes a new level of pressures and specificity not found in many systems where local adaptation has been studied, this research is a starting point for understanding emerging viral infectious diseases, which are currently some of the most threatening pathogens to wildlife biodiversity and population stability, as well as to the health and wellbeing of human populations globally.

Introduction

Emerging infectious diseases are increasingly appreciated in their effects on populations, communities and even on biodiversity (McCallum and Dobson 1995; Rachowicz et al. 2005; deCastro and Bolker 2005). EIDs, defined as diseases recently increasing in range, incidence or virulence, are a source of concern both in human and wildlife populations (Daszak et al. 2000). Wildlife EIDs can pose a threat to the stability and persistence of populations (Hudson et al. 2001; Rachowicz et al. 2005), sometimes even causing extinction (deCastro and Bolker 2005). Disease emergence is facilitated by some form of change in the host, pathogen, or both (Daszak et al. 2000). These changes include biological aspects such as rapid pathogen evolution and environmental changes that make available new hosts for diseases (Moren et al. 2004, Williams et al. 2002), as well as anthropogenic factors including human-mediated range expansions of infectious agents and increased density of both human and wildlife populations (Krause 1994, McCallum and Dobson 1995, Hess 1996). EIDs present challenges in terms of understanding the action of the infecting agent, the host response, and the interaction of the two.

Examining the extent to which host and pathogens are coevolved is essential for understanding disease dynamics (Jones et al. 2008), and particularly for EIDs. For example, recent host switches may result in pathogens being highly virulent due to a lack of coevolution (Storfer et al. 2007). Alternatively, hosts and pathogens are often thought of as being in an

"evolutionary arms race" and may experience cycles whereby there is cycling between pathogen infectivity and host resistance (Anderson and May 1982; Gomulkiewicz et al. 2000; Forde et al. 2004; Thompson 2005). Large population sizes and high mutation rates of pathogens generally results in the prediction that pathogens are locally adapted, with higher performance on source hosts than allopatric hosts (Kaltz and Shykoff 1998; Dybdahl and Storfer 2003). It has also been proposed that pathogen performance is negatively correlated with genetic distance from the source host (Ebert 1994). These patterns are likely due to performance tradeoffs from increased specialization on sympatric hosts that result in decreased performance on allopatric hosts (Clarke 1979, Kaltz and Shykoff 1998).

The mechanisms underlying these patterns may be genetic interactions of hosts and pathogen, with several hypotheses proposed regarding how host and pathogen genomes can undergo reciprocal selection. The matching allele model, for example, suggests that a pathogen allele must match a host allele for infection to occur (Agrawal and Lively 2002). That is, the pathogen can infect the host if there is a match of the susceptibility allele of the host and the infectivity locus of the pathogen. This model is thought to be most appropriate for viral systems because viruses often have envelope proteins which must match certain host cell receptors for the virus to invade the cell and replicate.

An important determinant of local adaptation is that there is spatial variation in the intensity of reciprocal selection in coevolutionary systems as in the geographic mosaic model (Gomulkiewicz et al. 2000, Nuismer et al. 2000, Lively et al. 2004, Thompson 2005). In some areas, coevolving species may exhibit strong reciprocal selection, potentially leading to coevolutionary arms races, whereas in other areas, selection may be weak or even absent due to non-overlap in species' ranges (Thompson 2005).

The tiger salamander, *Ambystoma tigrinum*, and its emerging viral pathogen *Ambystoma tigrinum* virus (ATV) have been extensively studied in recent years, providing key preliminary data for examining genetic interactions in natural populations. ATV is a double-stranded DNA virus with a large genome (~106 kb) that induces symptoms such as hemorrhaging, edema, skin sloughing and cloacal discharge in infected salamanders (Jancovich et al. 1997; Brunner et al. 2004). Additionally, some strains of the virus can result in extremely high mortality rates of infected salamanders (Bollinger et al. 1999, Jancovich et al. 2001, Brunner et al. 2005). One aspect that makes this system particularly useful for studying local adaptation is that in addition to availability of the genome sequence (Jancovich et al. 2003), variation in selection on three putative viral pathogenicity genes throughout western North America has been documented (Ridenhour and Storfer 2008). An eif2α gene is under purifying selection across all viral strains examined, a β-hydroxysteroid-oxidoreductase (β-OH) is under positive selection in certain clades of virus strains, whereas a CARD-caspase gene is under purifying selection in particular clades (Ridenhour and Storfer 2008, Table 1, Fig 1). However, some virus strains demonstrate an apparent lack of selection or frame shift mutations in the two latter genes, both of which may have altered the function of the particular virus strains with these mutations (Ridenhour and Storfer 2008).

The putative function of three of these pathogenicity genes is known (Chinchar 2002). The CARD caspase gene functions along apoptotic pathways and thus acts in limiting antiviral host responses (Hiscott et al. 2006, Johnson & Gale 2006). The eif2α gene functions in regulation of host translation and interferon downregulation (Essbauer et al. 2001). Finally, β-hydroxy steroid-oxidoreductase (β-OH) functions to up-regulate hormonal steroid synthesis, which is known to repress leukocyte activity and thereby mitigate host immune responses (Majji

et al. 2006). Knockout experiments of the latter two genes resulted in reduced viral virulence (Essbauer et al. 2001; Reading et al. 2003; Majji et al. 2006) thereby confirming their role in viral pathogenesis.

Microarray analysis of ATV infected individuals from the the axolotl, (*Ambystoma mexicanum*) which is a sister taxon to *A. tigrinum*, generally showed a lack of a T-cell response (Stewart et al. 2008), which could be a reason for observed high virulence and mortality rates caused by ATV (Jancovich et al. 1997, 2005). In addition, particular transcriptional patterns in the host seemed to reflect activity of viral virulence genes. For example, a CARD-caspase gene in the host which is involved in apoptotic pathways is down-regulated in response to the virus (Stewart et al. 2008). Up-regulation of apoptotic genes in response to viral infection is normally expected because apoptosis is one of the primary ways to kill infected host cells to prevent further viral replication (Jerome et al. 1999).

Given these background data, we performed a fully factorial infection study with four strains of the virus and each virus' sympatric subspecies of *A. tigrinum*. The viral strains each represented a different selection regime across the three genes (see Table 1). We predicted that the selective regime of the virus might determine pathogen local adaptation. Specifically, we predicted that the two populations under positive selection at the beta-hydroxysteroid gene (Donut Tank and Cap Pond strains) are not likely to not be locally adapted. Positive selection indicates that these populations are not at an adaptive peak because new non-synonmous mutations have been accumulating in the past. Hence, these populations may perform better on an "away" host, or that some foreign virus strain may perform better on these hosts. The Yellowstone virus is difficult to predict due to the frameshift mutations that may have altered the function of these virulence genes. However, this may also lead to local adaptation if these genes

are altered in function, they may not perform similarly on alternative hosts. Finally, the two virulence genes are not under selection in the Dalmeny population, suggesting that this population is locally adapted at a fitness peak on its sympatric population.

We tested these hypotheses in four ways: statistically comparing viral loads as an estimate of within host growth of each viral strain on its sympatric host versus all other allopatric hosts, by testing for correlations of viral growth with genetic distance of hosts, and by performing both home versus away and local versus foreign tests for local adaptation (Morgan et al. 2005). Home versus away looks for local adaptation by comparing viral performance across hosts, asking if a virus performs best on its source host. The local versus foreign analysis compares performance of all viral strains within a single host, predicting that for a given host the sympatric virus should have the highest performance if local adaptation is occurring (Kawecki and Ebert 2004). Through all of these estimates, we intend to determine if is the ATV pathogen is local adapted to its *A. tigrinum* host.

Methods

Animal collection rearing and experimental design

In Summer, 2007 larval *A. tigrinum nebulosum, mavortium, melanostictum* and *diaboli* were collected from sites in Arizona, Colorado, Wyoming and Saskatchewan, respectively (Fig. 1). Animals were returned to Washington State University where they were reared in individual 3 quart Ziploc containers in 500 ml aquifer water treated with ReptiSafe™, aerated for 24 hours, and changed weekly. The animals were reared in an environmental chamber at 20 ˚C on a 12:12 h light/dark cycle until approximately 4.5 months post-hatching, the same age at which previous microarray studies were conducted (Stewart et al. 2008). At this point, treatment animals were injected with 100 µL of 10^6 p.f.u. virus, whereas control individuals were injected with 100 µL

of cell culture medium (Eagle's minimum essential medium). This was 4 (host subspecies) x 5 (virus treatments 4 + control) factorial design experiment. Each viral strain was sympatric with one of the subspecies of salamander collected. The strains used were Donut Tank virus (DOT), Cap pond virus (CAP), Yellowstone virus (YEL) and Dalmeny virus (DAL, Fig. 1). There were 20 replicates, with individually-housed salamanders as the unit of replication per treatment combination, and 20 treatment combinations for a total of 400 animals. During the course of the experiment, behavioral observations were taken opportunistically to determine whether symptoms of infection were apparent.

At each of 72 and 144 hours post-infection, respectively, 200 animals were euthanized in MS222, and spleen and lung tissue was extracted and immediately frozen on liquid nitrogen. Spleen was selected due to its importance in the response to ranaviruses (Morales and Robert 2007, Stewart et al. 2008) and for immune system responses in general (e.g., lymphocyte proliferation). Lung tissue, was extracted because previous work suggests it is reliable for quantifying early-stage viral load (Stewart et al. 2008).

Molecular methods and statistical analyses

Genomic DNA was extracted from lung tissue using Qiagen DNeasy 96™ kits for estimates of viral load (Qiagen, Inc. CITY, STATE). Viral load of all individuals was estimated utilizing quantitative real time PCR (qPCR) according to the protocol detailed in Forson and Storfer (2006). A two-way ANOVA was performed to test for effects of viral strain, subspecies or a virus by subspecies interaction on viral load. In cases of significant main effects, pairwise comparisons were tested with Fisher's LSD.

Unpaired t-tests assuming equal variances were performed to test whether there was a significant difference in viral load for each virus strain on sympatric versus allopatric host

strains. To test whether within host growth was negatively correlated with host genetic distance, I performed two analyses. First, an estimate of viral growth was done by calculating the slope of a line fitted to the average viral load across time points for each treatment combination. Second, this slope was then regressed for each of the four virus strains against the genetic distance of each host from the source. Home versus away was calculated for each strain as the average sum of the difference between mean viral load in the local host minus mean viral load across each of the other host populations. Local versus foreign was calculated as the mean sum of the difference between viral load in the local host minus each of the viral loads of all foreign virus strains on that same host (Morgan et al. 2005). Finally, to get an overall assessment of local adaptation, a binomial test was performed for each strain on all of the measures taken.

Results

Observation of behavior and gross symptoms revealed three *A. t. diaboli* individuals infected with the YEL strain of the virus displayed symptoms at 144 hours including edema, hemorrhaging in the extremities, and cloacal exudate.

At 72 hours, the two way ANOVA revealed a significant effect of subspecies ($F_{3,128}$=2.9, p=0.04), viral strain ($F_{3,128}$=7.05, p=0.0002) and subspecies by strain interaction ($F_{9,128}$=3.5, p=0.0007) on viral load. *A. t. mavortium* infected with its sympatric virus, Cap Pond virus, had the highest average viral loads. Further analysis with a Fisher's LSD for all possible comparisons of subspecies and strain at this time reveals that all comparisons of viral load with the CAP virus and *A. t. mavortium* subspecies treatment combination were significant. Similarly, mean viral loads in *A. t. mavortium* differed from all other subspecies (*A. t. nebulosum, melanostictum,* and *diaboli*). The significant pairwise comparisons between strains were when Cap Pond strain was compared to any other strain (DOT, YEL and DAL, Tables 2

and 3). At 144 hours, however, the only significant main effect on viral load was subspecies ($F_{9,131}$=6.82, p=0.0003). (Table 4).

The t-tests comparing viral load of hosts infected with sympatric versus allopatric strains revealed differences between 72 and 144 hours post-infection. At 72 hours, the Cap Pond strain had significantly higher viral loads on its sympatric host versus all other hosts, suggesting local adaptation (p=0.03, Fig 3). However at 144 hours, the Dalmeny strain had significantly higher viral loads on its sympatric host than all other hosts (p=0.003, Fig 4). The regressions of genetic distance and viral growth revealed no significant correlations, although the r^2 values for Donut Tank and Yellowstone viruses suggest a correlation but with four data points power to detect these correlations was low (Fig 5).

The home versus away analyses revealed a slightly different pattern of local adaptation between time points. At 72 hours, Cap Pond and Dalmeny showed evidence of local adaptation as indicated by positive values, whereas Yellowstone and Donut Tank showed evidence of maladaptation. At 144 hours Cap Pond still showed evidence of local adaptation and Yellowstone still showed evidence of maladaptation. However, Dalmeny at 144 hours appears to be maladapted, and DOT appears to be locally adapted (Table 5).

Similarly, the local versus foreign analysis appears to show different results between the two time points. Cap pond virus is locally adapted at both 72 and 144 hours, and Dalmeny virus is maladapted at both time points. However, Donut Tank virus is maladapted at 72 hours and locally adapted at 144 hours, and Yellowstone virus is locally adapted at 72 hours and maladapted at 144 hours. Results of all four analyses performed are found in Table 6. The binomial test on each strain indicates that only the Donut Tank strain was significantly maladapted (p=0.003).

Discussion

Our examination of the performance of four strains of *Ranavirus* on their allopatric and sympatric host subspecies of *Ambystoma tigrinum* revealed possible local adaptation of one viral strain and evidence of maladaptation of another strain. The Donut Tank and Cap Pond viruses were predicted to not be locally adapted due to the presence of positive selection, whereby favorable mutations would spread if they conferred an adaptive benefit. The results for Donut Tank indicate it is significantly maladapted across all measured examined. These results are consistent with the hypothesis that because this virus is under the strongest overall selection, that it should be the least well adapted. The Cap Pond virus is under less intense selection and appears locally adapted which would fail to support our hypothesis, although the binomial test reveals it is non-significant. This could indicate that either the positive selection on the β-hydroxy-steroid gene is not essential in this case for local adaptation, or that other genes not investigated are under selection. That is, there are seven other known virulence genes in ATV (Jancovich et al. 2003), and complex patterns of selection across these genes may affect this particular virus-host interaction. .

The Yellowstone virus appears to be locally maladapted, potentially due to frame shift mutations in the two putative virulence genes examined. However, further research should assess whether knockout mutants that lack these genes or additional strains where these genes are translocated perform. Clearly, however, the frameshift mutations did not render this virus strain dysfunctional, but rather affected its ability to match its local host population. It is interesting to note that this virus has overall the lowest viral loads at the 72 hour timepoint, but viral loads increased at a rate greater than all other strains between 72 and 144 hours.

Finally, the Dalmeny virus was predicted to be locally adapted because the lack of selective pressures resulting in the assumption this virus is near or at its fitness optimum. However, five of our eight measures suggest maladaptation, although the binomial test was not significant. It is also a possibility that, while there is no apparent selective on the genes examined, the virus may perform well on alternate hosts.

It is noteworthy that all of the measures calculated in this experiment appear to give mixed results for the viral strains. If one were to observe only the home versus away and local versus foreign measures for the CAP viral strain, it would appear to be locally adapted, but further analysis indicates that it is equivocal. More measures or other alterations to the experimental scheme could prove useful in determining if this virus is truly maladapted or if this is an artifact of the experiment itself. For instance, the animals were injected with virus in this case to ensure infection of the host, but in the future experiments utilizing more ecologically relevant means of virus exposure, such via water bath, could yield different results.

To more fully examine this local adaptation or coevolutionary dynamic between host and pathogen, in the future it will be necessary to incorporate host factors that have not previously been examined in *A. tigrinum*. Examining host transcriptional responses across treatment combinations, or examining the responses of the host at a proteomic or immunological level would also prove insightful, as coevolution is an interaction between host and pathogen and these host factors play an enormous role in determining the outcome of viral infection. While we have a baseline of knowledge now regarding the transcriptional changes occurring during infection with the virus due to the research performed in chapter one, expanding this knowledge to include whether the virus is affecting immunologic function in the host (Robert et al. 2005, Maniero et al. 2006) or otherwise altering host responses between sympatric and allopatric hosts

would provide a more complete picture of the interactions that are occurring. For instance, we are currently lacking understanding of T-cell responses to the virus, and exact immunologic function of the innate immune response, such as whether interferon is actually being produced or if it is just coded for in the transcriptomic response.

Host-pathogen systems are dynamic, continuously changing their interactions as host-pathogen interactions often lead to reciprocal selection. As the variability in these interactions drives both coevolution itself (Thompson 1994) and biological diversity (Thompson 1999), understanding this variation can lead to a better insight into both. This geographic variation in selection and its resulting reciprocal selection drives ecological patterns (Thompson 1999). The study of coevolution unites disciplines ranging from epidemiology to conservation and this has helped to make evolutionary ecology a central discipline in the sciences (Thompson 1996, 1997, 1998, 1999).

Finally, our research is novel in that it presents one of the first studies of local adaptation of a virus on a vertebrate host. There are currently several global EIDs of humans that are viral, such as West Nile Virus, H5N1 Influenza, and Taylor et al. (2001) determined that viruses composed 44% of the emerging human pathogens at that time. Due to the fact that viruses emerge so readily, understanding how viral pathogens not only emerge, but coevolve with vertebrate hosts is essential. Knowledge of how the complexities of the vertebrate immune system alter this interaction can alter strategies to control viral pathogens or determine their spread. For example, being able to determine if a virus will perform better on a source or non-source host is an important piece of information in determining the risk of disease spread and, in the cases of zoonoses, the risk of the pathogen being spread to alternate hosts and being spread in that manner. Therefore, this research can be used as a launching point in understanding how the

vertebrate immune system affecting this coevolutionary dynamic, how it is leading to or preventing local adaptation, and the implications this has for the spread of an emerging disease.

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Table 1. Comparison of selection type on the infecting viral strains broken down by virulence gene. Shows the entire selection profile known for the four viral strains utilized in the experiment.

	Viral Strain			
Gene	*Donut Tank*	*Cap Pond*	*Yellowstone*	*Dalmeny*
eif2α	Purifying	Purifying	Purifying	Purifying
CARD	Purifying	None	Frameshift	None
β-OH	Positive	Positive	Frameshift	None

Table 2. All possible pairwise comparisons of viral load by *A. tigrinum* subspecies at 72 hours post-infection. Significant p-values (adjusted with a Fisher's LSD) are denoted in bold

	A. t. diaboli	A. t. mavortium	A. t. melanostictum	A. t. nebulosum
A. t. diaboli	----	**0.03**	0.83	0.76
A. t. mavortium		-----	**0.02**	**0.01**
A. t. melanostictum			------	0.93
A. t. nebulosum				------

Table 3. All possible pairwise comparisons of viral load by viral strain at 72 hours post-infection. Significant p-values (adjusted with a Fisher's LSD) are denoted in bold.

	Cap pond virus	Yellowstone virus	Dalmeny virus	Donut Tank virus
Cap pond virus	-----	**<.0001**	**0.03**	**0.0002**
Yellowstone virus		------	0.07	0.76
Dalmeny virus			-----	0.12
Donut Tank virus				------

Table 4. Pairwise comparisons of viral load by *A. tigrinum* subspecies as estimated by quantitative real-time PCR at 144 hours post-infection. Significant p-values (adjusted with a Fisher's LSD) are denoted in bold.

	A. t. diaboli	A. t. mavortium	A. t. melanostictum	A. t. nebulosum
A. t. diaboli	----	**0.001**	**0.001**	0.444
A. t. mavortium		-----	0.949	**0.013**
A. t. melanostictum			------	0.476
A. t. nebulosum				------

Table 5. Home versus away and local versus foreign analyses for 72 and 144 hour time points. A positive value indicates local adaptation, whereas a negative value indicates a lack of local adaptation for that viral strain.

	Local versus Foreign		Home Versus Away	
	72 hours	144 hours	72 hours	144 hours
Cap Pond	+	+	+	+
Donut Tank	-	-	-	-
Dalmeny	-	+	+	-
Yellowstone	+	-	-	+

Table 6. Outcome of all four local adaptation measures for each viral strain at both time points. R= Regression of genetic distance against viral growth. A = t-test comparison of mean viral load of allopatric versus sympatric hosts. H = Home versus away estimate. L= Local versus foreign estimate. (+) indicates local adaptation, (-) indicates no local adaptation.

Virus	Time Point	Measure				
CAP		R	A	H	L	B
	72 hours	-	+	+	+	0.219
	144 hours	-	-	+	+	
DOT						
	72 hours	-	-	-	-	0.004
	144 hours	-	-	-	-	
DAL						
	72 hours	-	-	+	-	0.219
	144 hours	-	+	-	+	
YEL						
	72 hours	-	-	-	+	0.109
	144 hours	-	-	+	-	

Figure 1. Map of tiger salamander subspecies and their sympatric viral strains, along with selective pressures on clades of the virus. Viral strains with boxes around them are strains utilized in the experiment. Figure adapted from Storfer and Eastman.

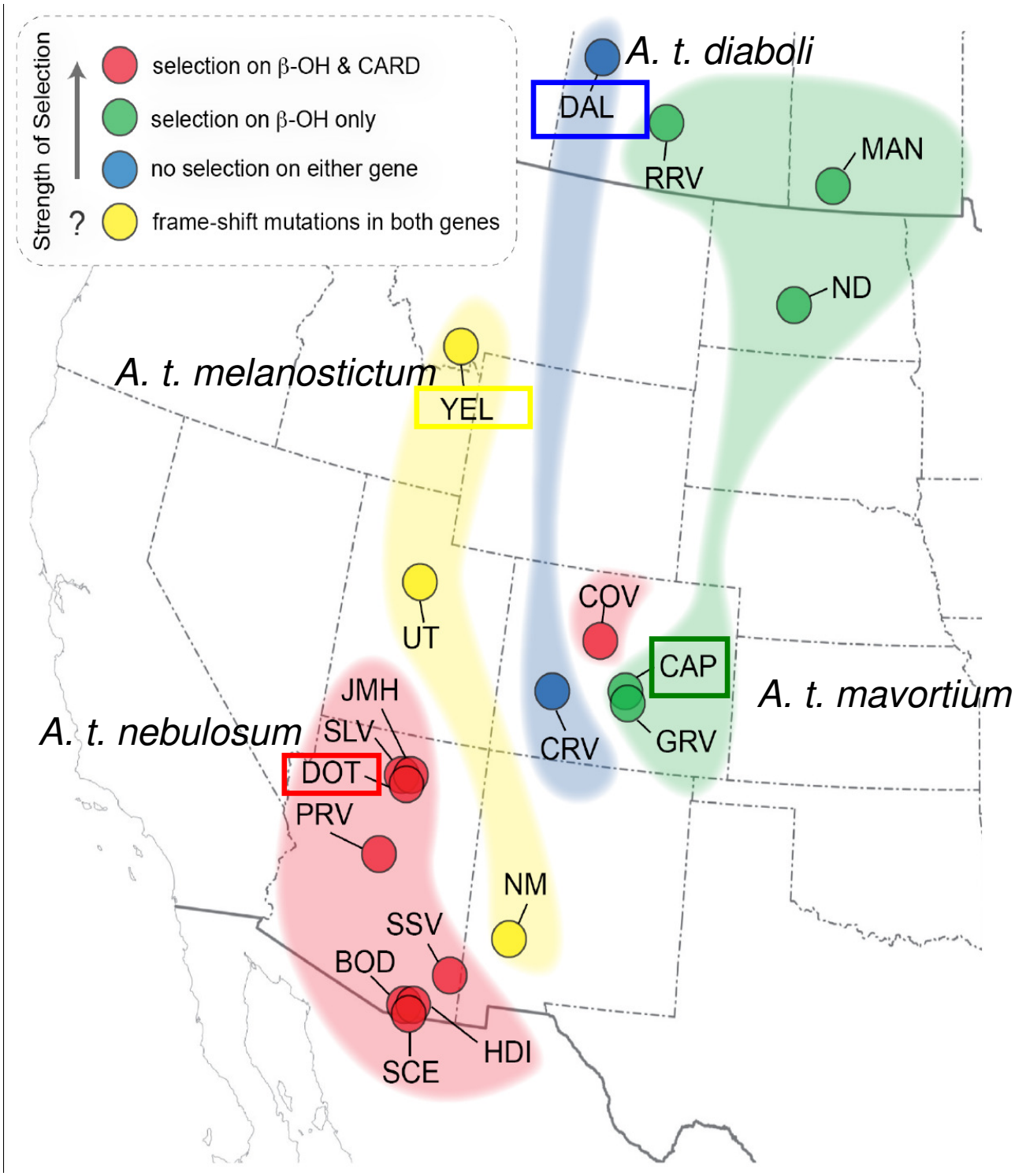

Figure 2. Mean viral loads at 72 and 144 hours for each strain of the virus across all hosts. A. 72 hour viral loads. B. 144 hour viral loads.

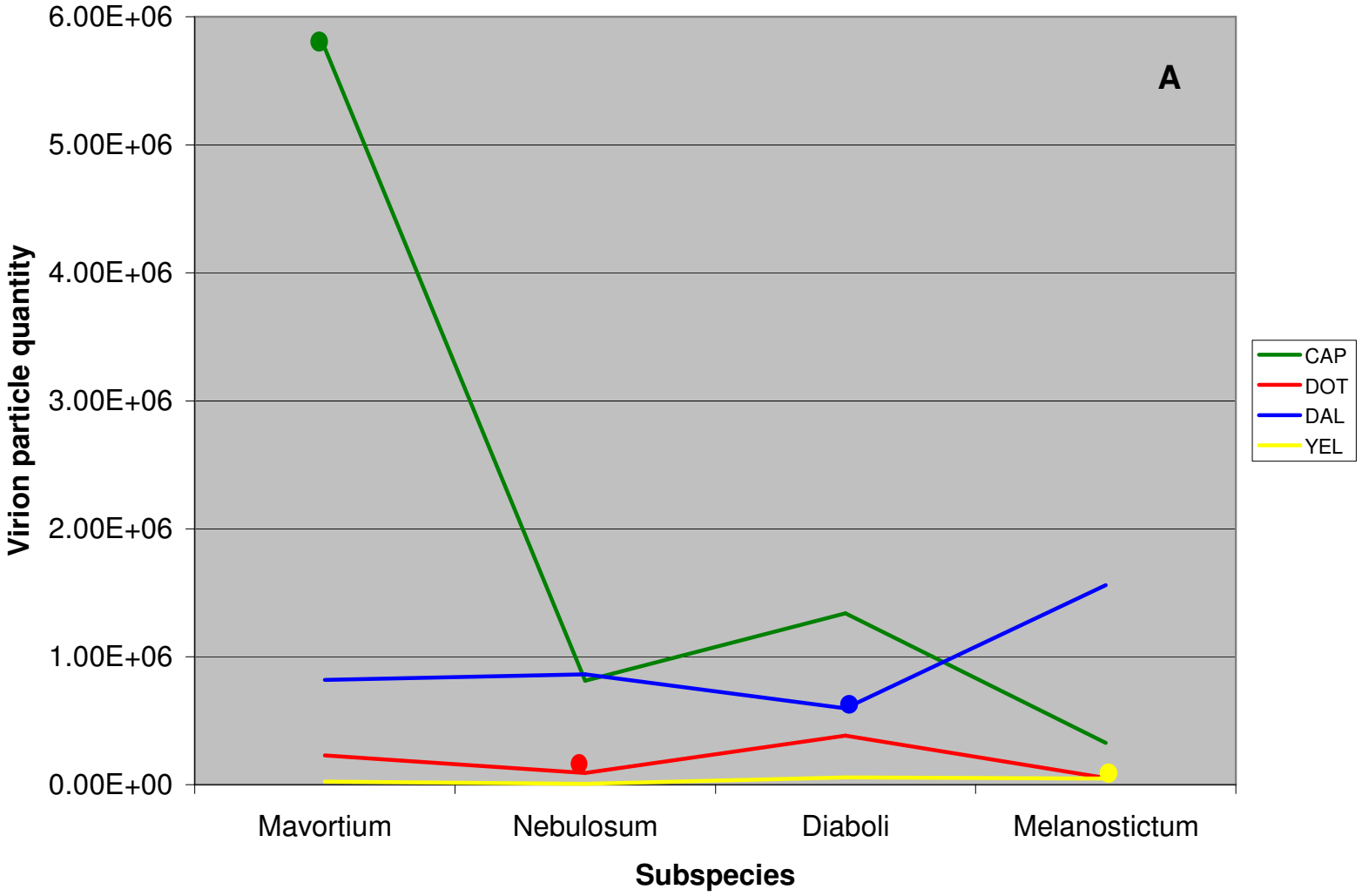

Figure 2, cont'd

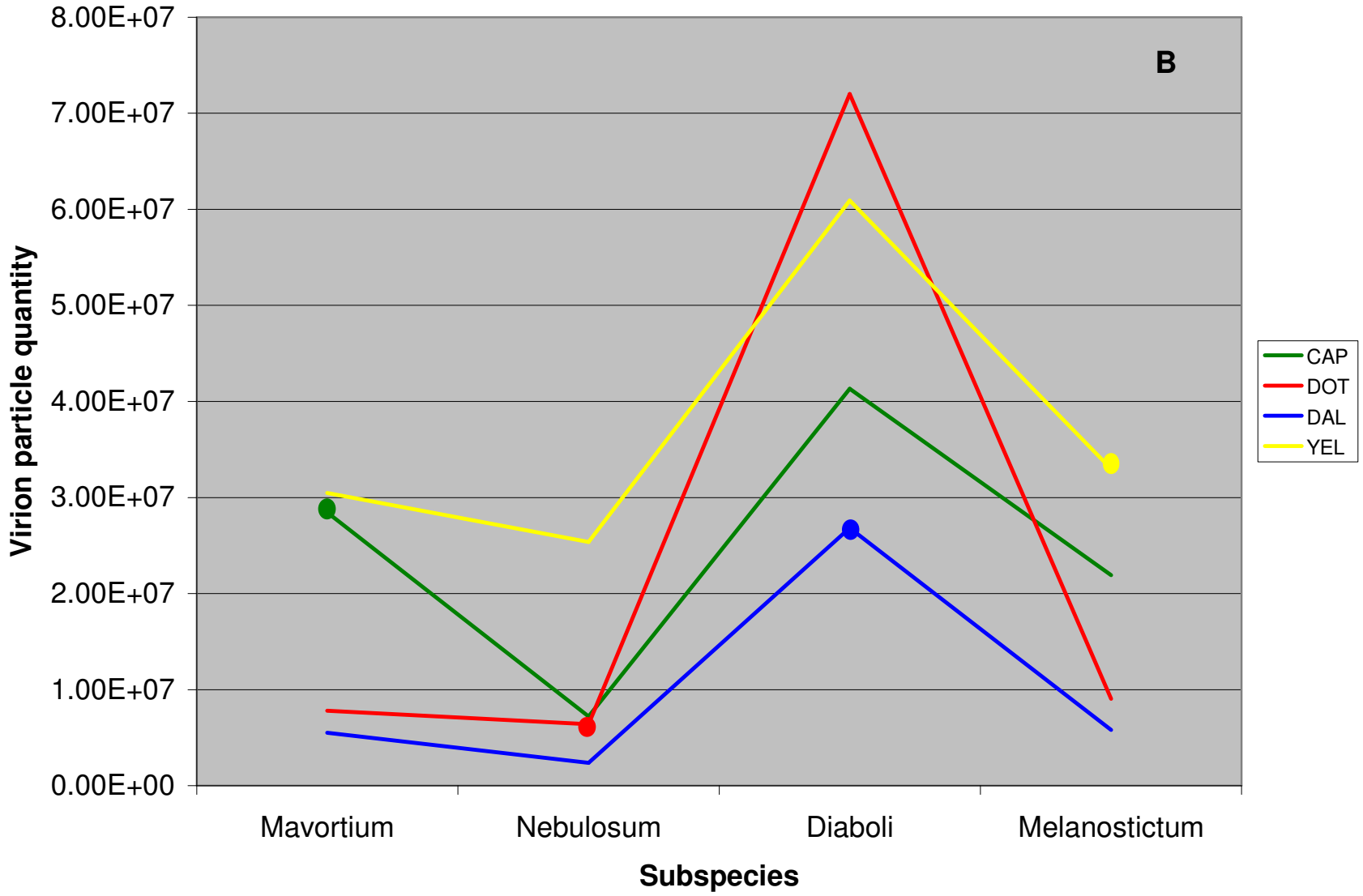

Figure 3. Comparison of the log of viral load for each strain at 72 hours post-infection. The mean viral load of a given viral strain on its sympatric host is indicated with a black bar, where the mean viral load in all other allopatric hosts is indicated in white. An (*) indicates a significant comparison according to an unpaired t-test.

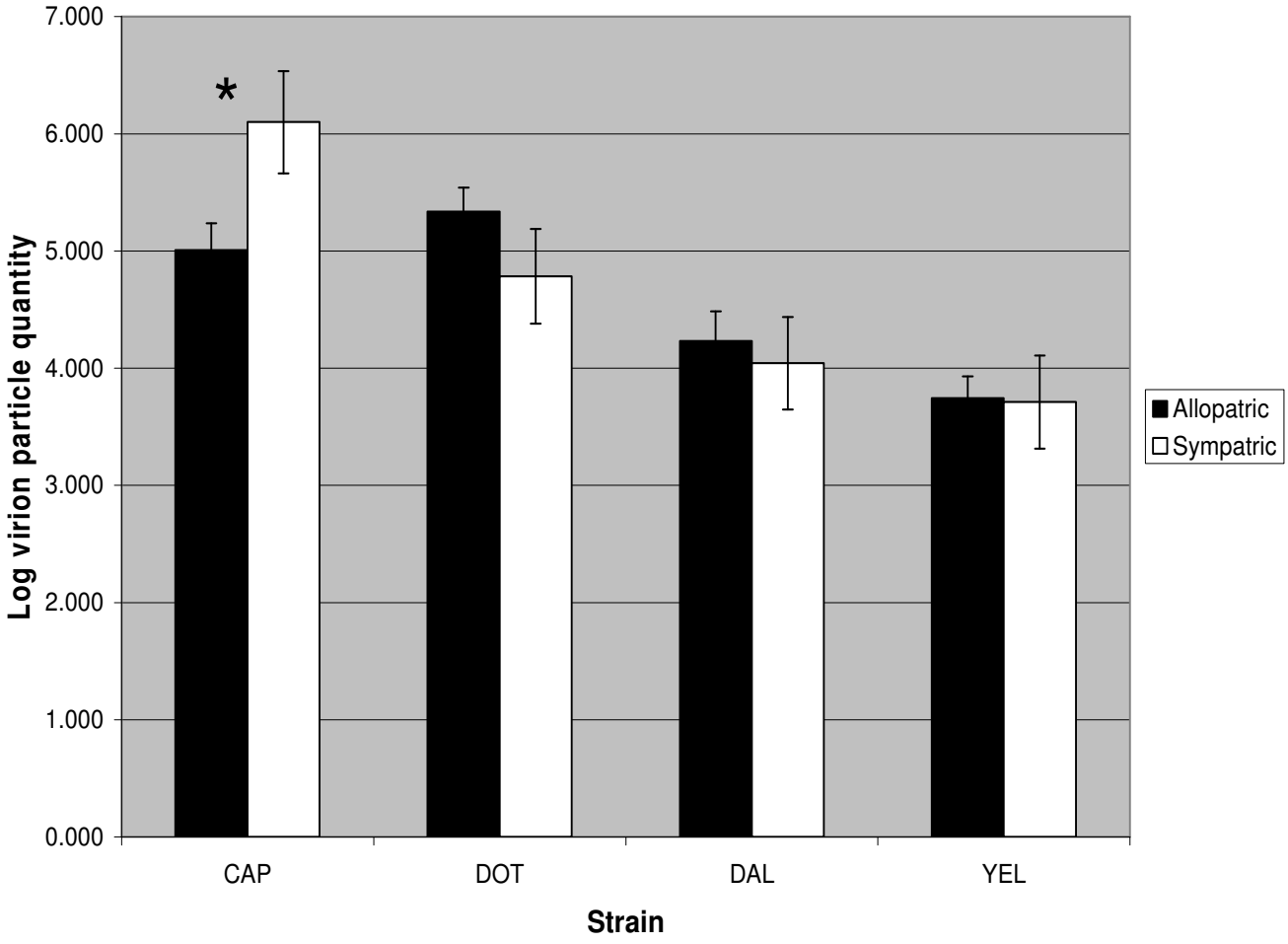

Figure 4. Comparison of the log of viral load for each strain at 144 hours post-infection. The mean viral load of a given viral strain on its sympatric host is indicated with a black bar, where the mean viral load in all other allopatric hosts is indicated in white. An (*) indicates a significant comparison according to an unpaired t-test.

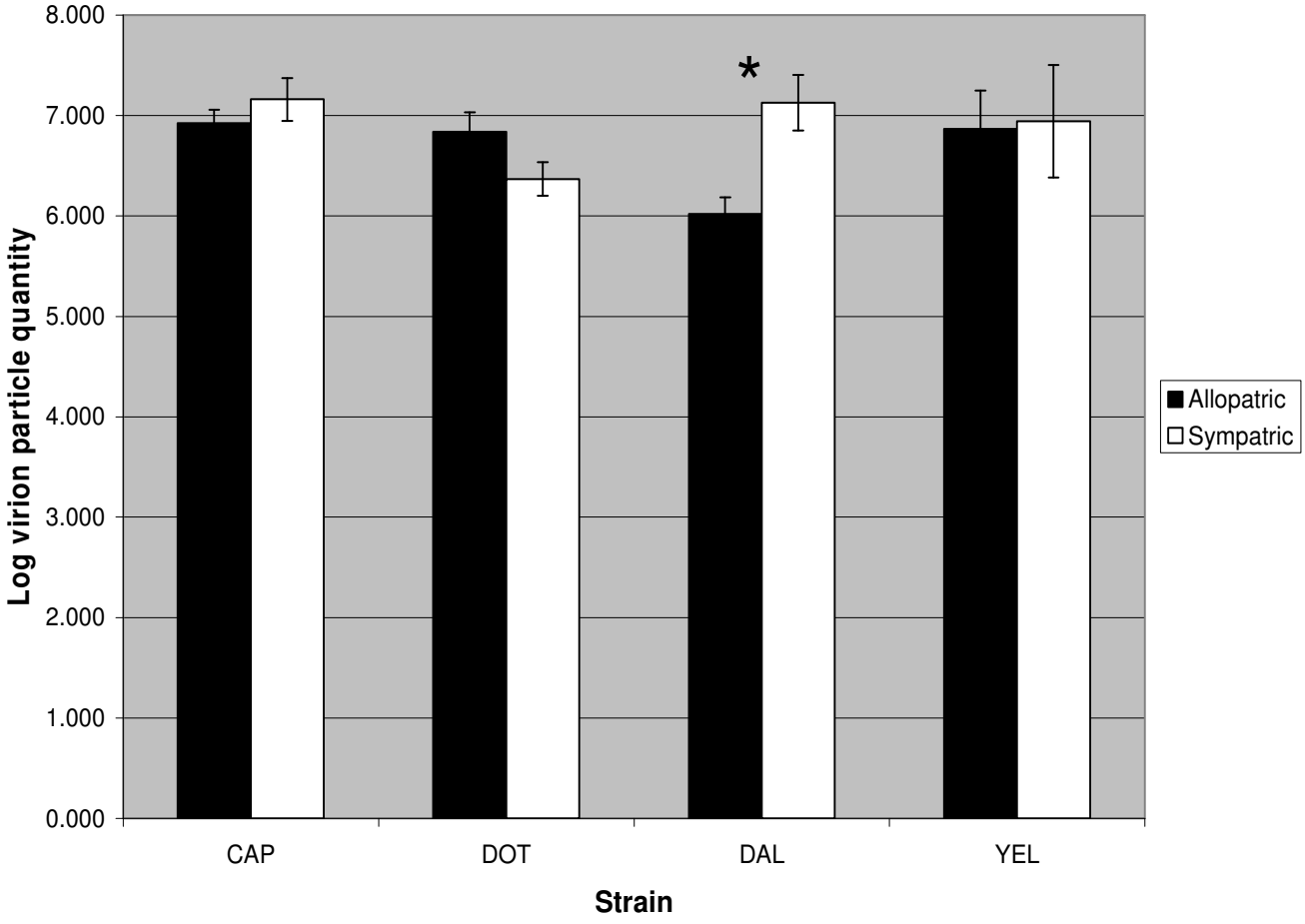

Figure 5. The regression of host genetic distance against the slope of within-host viral growth between 72 and 144 hours. P-values and r^2 values are presented within each figure. A. Cap Pond strain. B. Dalmeny strain. C. Donut Tank Strain. D. Yellowstone Strain