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Metagenomic Analysis Indicates that Stressors Induce Production of Herpes-Like Viruses in Coral *Porites compressa*

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At the time of this publication Dr. Barott was affiliated with San Diego State University, but she is now a faculty member of the University of Pennsylvania.

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Abstract
During the last several decades corals have been in decline and at least one-third of all coral species are now threatened by extinction. Coral disease has been a major contributor to this threat, but little is known about the responsible pathogens. To date most research has focused on bacterial and fungal diseases; however, viruses may also be important for coral health. Using a combination of empirical viral metagenomics and real-time PCR, we show that *Porites compressa* corals contain a suite of eukaryotic viruses, many related to the Herpesviridae. This coral-associated viral consortium was found to shift in response to abiotic stressors. In particular, when exposed to reduced pH, elevated nutrients, and thermal stress, the abundance of herpes-like viral sequences rapidly increased in 2 separate experiments. Herpes-like viral sequences were rarely detected in apparently healthy corals, but were abundant in a majority of stressed samples. In addition, surveys of the *Nematostella* and *Hydra* genomic projects demonstrate that even distantly related Cnidarians contain numerous herpes-like viral genes, likely as a result of latent or endogenous viral infection. These data support the hypotheses that corals experience viral infections, which are exacerbated by stress, and that herpes-like viruses are common in Cnidarians.

Keywords
coral reefs, disease, Herpesviridae, viral-like particles, virome

Comments
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Metagenomic analysis indicates that stressors induce production of herpes-like viruses in the coral *Porites compressa*

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During the last several decades corals have been in decline and at least one-third of all coral species are now threatened with extinction. Coral disease has been a major contributor to this threat, but little is known about the responsible pathogens. To date most research has focused on bacterial and fungal diseases; however, viruses may also be important for coral health. Using a combination of empirical viral metagenomics and real-time PCR, we show that *Porites compressa* corals contain a suite of eukaryotic viruses, many related to the Herpesviridae. This coral-associated viral consortium was found to shift in response to abiotic stressors. In particular, when exposed to reduced pH, elevated nutrients, and thermal stress, the abundance of herpes-like viral sequences rapidly increased in 2 separate experiments. Herpes-like viral sequences were rarely detected in apparently healthy corals, but were abundant in a majority of stressed samples. In addition, surveys of the *Nematostella* and *Hydra* genomic projects demonstrate that even distantly related Cnidarians contain numerous herpes-like viral genes, likely as a result of latent or endogenous viral infection. These data support the hypotheses that corals experience viral infections, which are exacerbated by stress, and that herpes-like viruses are common in Cnidarians.

coral reefs | disease | Herpesviridae | viral-like particles | virome

Coral disease is a major cause of the pandemic decline of tropical reefs (1, 2). Increases in coral disease incidence have occurred during the last several decades (3, 4), and, although their devastating effects on coral populations are no longer in question (5), in most cases, it is not yet clear whether specific or opportunistic pathogens are involved (6, 7).

A challenge to identifying causative agents of coral disease is that corals naturally harbor a variety of microbiota including symbiotic algae, fungi, Bacteria and Archaea (8–13). Corals demonstrate species-specific and potentially mutualistic relationships with communities of Bacteria (14), and these associated communities are altered during episodes of coral disease (7, 15). To date, no coral viral diseases have been positively identified, but pathologies, such as yellow blotch and white band disease, have been hypothesized to be the results of viral infection (16).

Virus-like particle abundances shift both across reefs and with proximity to coastal areas and corals themselves (17, 18). On tropical reefs viral numbers range from 0.3 to 1.25 $\times$ 10^7 particles per milliliter in reef water (19) to 0.8–1 $\times$ 10^9 particles per cubic centimeter within the sediment (20). Small-scale variations in viral numbers occur with changing proximity to coral colonies, with viral particles peaking ≈2–4 cm above the coral surface. These abundances are elevated in waters surrounding diseased coral colonies from 1.0 $\times$ 10^7 to 1.5 $\times$ 10^7 particles per milliliter (21). Virus-like particles (VLPs) also occur within corals and their relatives. For example, VLPs have been found within the tissues of anemones and within the symbiotic zoochlorellae and zooxanthellae of various Cnidarians (22–24). While the identity of these VLPs has yet to be established, an increase in viral particles within dinoflagellates has been hypothesized to be responsible for symbiont loss during bleaching (25–27). VLPs also have been identified visually on several species of scleractinian corals, specifically: *Acropora muricata*, *Porites lobata*, *Porites lutea*, and *Porites australiensis* (28). Based on morphological characteristics, these VLPs belong to several viral families including: tailed phages, large filamentous, and small (30–80 nm) to large (>100 nm) polyhedral viruses (29). Metagenomic data have also shown that both phages and eukaryotic viruses are associated with *Porites astreoides* (30) and healthy and bleaching *Diploria strigosa* corals (31).

Environmental stress often results in coral bleaching, disease, and death. Increased temperature, nutrient loading, dissolved organic carbon pollution, and reductions in ambient seawater pH are of particular concern due to their effects on the coral-symbiont relationship, host homeostasis, microbial overgrowth, and skeletal deposition (32–34). To determine whether environmental perturbations shift the eukaryotic viral assemblage present in corals, these 4 parameters were manipulated, and the resulting viral consortia characterized through the generation of 6 metagenomes. We show here that changes in the viral repertoire varied across each treatment, but all treatments contained genomic sequence similarities to specific eukaryotic viral families, most notably the *Herpesviridae*. Furthermore, a unique herpes-like viral sequence (HLVs) increased up to 6 orders of magnitude in coral specimens exposed to increased temperature, nutrients, and pH stress indicating viral production within the host. From these and other data, we hypothesize that a distantly related herpes-like virus or group of viruses is commonly associated with corals and Cnidarians in general and are produced as a result of stressor events.

Results and Discussion

Experimental Design and Rationale. To characterize coral-associated viruses, specimens of the finger coral, *Porites compressa*, were collected in the Hawai'i Marine Laboratory Refuge. This species of coral was chosen because it is a common and endemic species of coral in the Hawaiian archipelago and is the

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (genome project and accession nos. 28415, 28417, 28419, 28421, 28423, and 28425 and EU660955).

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dominant species of coral across reefs in Kane'ohe Bay where Coconut Island and the Hawai'i Institute of Marine Biology are located. Numerous studies have been conducted on this coral and there is a legacy of information on the oceanography and ecology of these reefs, providing pertinent data for insights into the viral ecology of corals across this region.

Coral specimens were exposed to thermal (5° C above ambient), dissolved organic carbon (25 mg·mL^{-1} glucose), nutrient (10 μM nitrate, nitrite, phosphate, and ammonium), and pH (1.7 units below ambient) stress for 64 hours [supporting information (SI) Fig. S1]. In brief, 3 corals were broken into fingers and placed in aquaria containing a given treatment. Coral samples or "nubbins" were collected just before the experiment ("time zero") to measure shifts in the viral consortia that resulted from isolation from the reef, and to provide several unadulterated samples for comparison. Subsequent samples were taken after 1, 4, 16, and 64 h of stressor exposure. Six viromes were generated by pooling 1 μg of DNA from each time point: time zero, "aquarium control," "temperature," "DOC," "pH," and "nutrient" (Fig. S1). Pyrosequencing was conducted at 454 Life Sciences on a GS20. Sequence reads were parsed and analyzed using bioinformatics.

Library Statistics and Bioinformatic Analysis. The sequenced viromes contained between 30,000 and 51,000 reads with an average length of 105 bases, a dataset of >25 million bases. Several bioinformatic techniques identified changes in the eukaryotic viral consortia across each library. Sequence reads were first compared using BLASTn to the nonredundant database at the National Center for Biotechnology Information (Table S1). A sequence was assigned as "known" if it had a similarity ($e \leq 10^{-4}$) to the nonredundant database. These low stringency parameters were chosen because viral DNA often has little similarity to known sequences in the non-redundant database (35). "Knowns" were then taxonomically sorted (viral, bacterial, archaeal, and eukaryotic) based on their highest similarity. In agreement with previous findings, these metagenomes contained few similarities to known viral sequences (Table S1) (36). A majority of sequences were found to be similar to bacterial and eukaryotic annotations. Analyses of 16S and 18S rDNA PCR and similarities to the human genome (see Methods) demonstrated that eukaryotic and microbial DNA contamination did not contribute to these sequences. This high percentage of known sequences similar to Bacteria and Eukaryota is likely the result of (*i*) protein conservation across viral, microbial, and eukaryotic genomes; (*ii*) the larger size and larger number of microbial genomes at the National Center for Biotechnology Information database; (*iii*) prophage and retrotransposon-like sequences within microbial and eukaryotic genomes; and (*iv*) the genetic mosaicism of viruses. The entire library was also compared with 4 other marine viromes (Table S2) to determine whether the coral viromes resembled seawater viruses. Few similarities were found between the coral viromes and water samples, suggesting that seawater contamination was not an issue.

Coral-Associated Viral Consortia Change with Experimental Manipulation. One goal of this study was to determine the types of eukaryotic viruses infecting the coral holobiont. Phage data will be presented elsewhere. As few sequences (Table S1) were found to be similar to any viral sequences at the National Center for Biotechnology Information, reanalysis of the metagenomic reads was conducted using tBLASTx ($e \leq 10^{-6}$) to a boutique database of the fully sequenced eukaryotic viral genomes from the National Center for Biotechnology Information database.

Metagenomic sequences with significant similarities ($e \leq 10^{-6}$) were assigned as "knowns" based on the sequence's best similarity (i.e., the top hit) to each reference viral genome. To compare and contrast the overall differences between each library, sequences were first sorted at the family level, and the percentage of known similarities to each viral family was calculated.

Of the ≈100 viral families described thus far (37), 19 were found

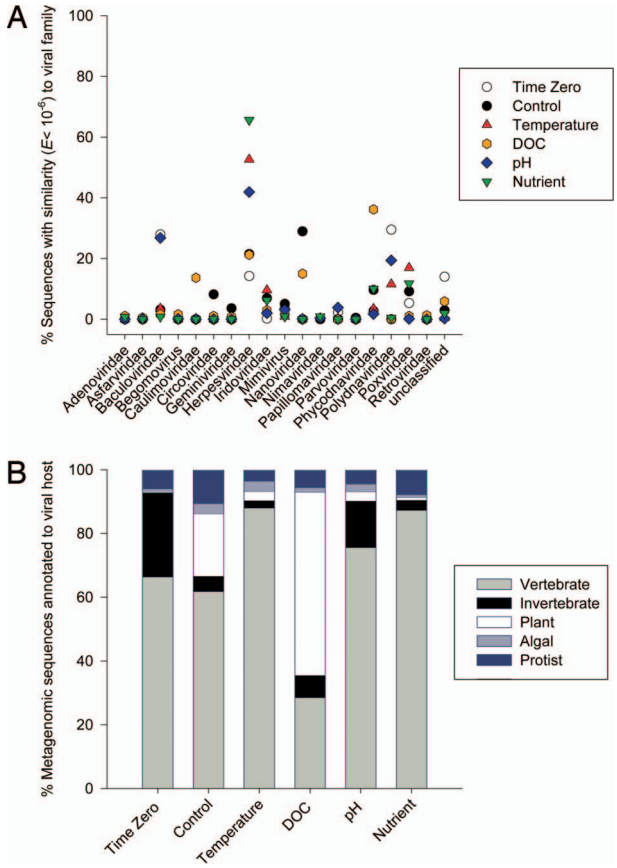

Fig. 1. A) Percentages of best similarities (tBLASTx, $e \leq 10^{-6}$) to 19 families of viruses in each individual coral-associated viral metagenome: Time Zero (open circles), Aquarium Control (filled circles), Temperature (red triangles), pH (blue diamonds), Nutrient (green arrows), and DOC (orange hexagons). (*B*) Predicated host ranges across each metagenome, with libraries containing vertebrate (light gray) and invertebrate (black bars) associated viral sequences and plant (white bars) and algal (dark-gray bars) viruses. Blue bars represent similarity to viral genomes that are associated with Protists.

in the combined coral-associated viromes (Fig. 1*A*). For example, the time zero samples contained 23% *Baculoviridae*-like and 30% *Polydnaviridae*-like sequences. *Baculoviridae*-like sequences also accounted for 23% of the similarities in the pH (blue diamonds) sample. The aquarium control (filled circles) contained 30% and the carbon-loaded DOC samples (orange hexagons) 18% sequences similar to the *Nanoviridae*. A large proportion of the best sequence similarities in the stressed samples were related to the *Herpesviridae* family, with the nutrient, pH, and temperature-perturbed samples containing 64%, 53%, and 70% herpes-like sequences, respectively.

Coral-Associated Viruses Infect a Range of Hosts. A discriminating characteristic in viral taxonomy is host range (38). Corals are a collective of cnidarian tissue, microbial symbionts, fungi, and other microeukaryotes. To determine the potential hosts of the coral-associated viruses, sequences were categorized according to a predicted generalized host range defined by the known host of the similar reference genome. These host ranges were modified from the Universal Virus Database from the International Committee on the Taxonomy of Viruses (37). Using these eukaryotic viral assignments, the predicted host range of the coral-associated viruses changed with each stressor treatment (Fig. 1*B*). In each of the viromes, >38% of the similarities were associated with vertebrate hosts, including representatives of most of the herpesviruses, poxviruses, adenoviruses, papillomaviruses, circoviruses, and iridovi-

Table 1. All sequence similarities ($e < 10^{-6}$) based on tBLASTx analysis to a boutique database containing the fully sequenced eukaryotic viral genomes from the National Center for Biotechnology Information database and the abundance and percentage of those related to herpesviruses (HV)

Sample	Sequences	Eukaryotic Virus	HV	HV, %
Time zero	39,270	11,826	2272	19.21
Control	39,340	414	81	19.57
Temperature	39,036	667	201	30.13
DOC	35,680	373	7	1.88
pH	50,368	11,831	3791	32.04
Nutrient	34,433	858	277	32.28

ruses (light gray bars). The Aquarium Control and DOC samples contained a large fraction of sequences similar to ssDNA viruses from plants (white bars) such as geminiviruses and nanoviruses (39). Invertebrate infecting viruses (black bars) were also abundant (>60%) within the time zero library and were mostly similar to the baculoviruses and polydnaviruses, 2 viral families that predominantly infect arthropods (40–42).

However, a caveat to this analysis is that the predicted host range is biased by the larger number of completed vertebrate-associated viral genomes. This analysis is also based on the sequences' best similarity, but almost certainly represents distant relatives of the reference viruses. These data suggest that coral-associated viruses possibly have many target hosts, including metazoans, protists, fungi, and plants.

Stressors Increase the Abundance of Herpes-Like Viral Sequences. Each virome sequence may have multiple similarities due to gene conservation. Therefore, each library was also analyzed for the total number of sequence similarities (as opposed to the best similarity in Fig. 1) to each of the eukaryotic viral genomes at the National Center for Biotechnology Information database, using tBLASTx ($e \leq 10^{-6}$). Individual viromes had between 300 and 11,000 predicted protein level sequence similarities to all eukaryotic viral genomes (Table 1). However, the number of these sequences that had homology to the *Herpesviridae* was different between the stressors. The percentage of all of the viral similarities to the *Herpesviridae* was similar in the time zero (≈19%) and the control (≈19%) but was elevated to 30%, 32%, and 32%, in the temperature, nutrient, and pH samples, respectively. The DOC sample had few sequences (< 2%) related to *Herpesviridae*.

The large abundance of *Herpesviridae* similarities in the temperature, pH, and nutrient samples indicated that these stressors induced production of viruses with distant relation to the herpesviruses. A caveat to this analysis, however, is that the short read length combined with low sequence homology limits taxonomic resolution (43). To verify if herpes-like viruses were present in the samples, sequences from all of the libraries were combined, compared using tBLASTx ($e \leq 10^{-6}$) to the eukaryotic viral boutique database, and then plotted along the corresponding reference genomes. The combination library contained both broad and deep coverage to some herpesvirus (44–48) genomes (Fig. 2 *A–C* and Table S3), but low coverage to other marine and cnidarian-associated viral genomes (Fig. 2 *E–H* and Table S3), such as a panaeid shrimp virus, a zoochlorella infecting phycodnavirus (2D), a phytoplankton virus (2E), and a fish iridovirus (2F) genome (49). From these analyses, we hypothesized that herpes-like viruses (HLVs) are present in corals and become more abundant as a result of stress events.

Real-Time PCR on Original Temporal Samples Confirms the Production of HLVs. Viral production, or the generation of new virions by the host, has been effectively quantified using real-time PCR to genes of various RNA and DNA viruses (50). To determine temporal changes in viral abundance across the manipulation experiments, metagenomic sequences were assembled, annotated, and given a taxonomic classification according to the sequence's best protein similarity using PSI-BLAST to the non-redundant database (see *SI Methods* for details). Sequences with significant homology ($e \leq 10^{-6}$) to herpesviruses were chosen, and primers were generated for both conventional and real-time PCR. One sequence was 62% identical ($e < 4 \times 10^{-11}$) to a single copy thymidylate synthase gene from *Saimiriine herpesvirus 2* (Fig. S2). This sequence was amplified, and the resulting PCR product cloned and Sanger sequenced to verify the assembly (GenBank accession no. EU660955). No differences were found between the original assembly and the cloned nucleotide sequence (data not shown).

These primers were used for real-time PCR, and it was found that the copy number of this putative herpes-like viral thymidylate synthase sequence increased during the original stressor experiment (Fig. 3*A*). The target sequence was below the detection level in all of the zero hour time points with the

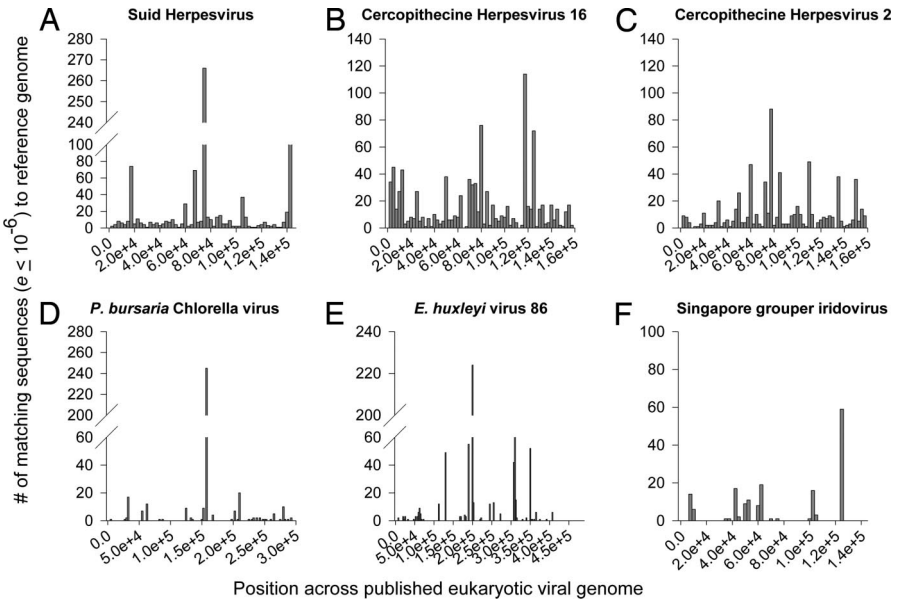

Fig. 2. Coverage of selected *Herpesviridae* genomes and other marine associated viral genomes. Libraries were combined and sequences analyzed for similarity (tBLASTx $e \leq 10^{-6}$) to a boutique database of 2,020 fully sequenced eukaryotic viral genomes collected from the National Center for Biotechnology Information. Genomes were separated into 2,500 base pair bins. (*A–C*) Broad and high fold coverage was found for many mammalian associated *Herpesviridae* genomes, such as a suid herpesvirus (*A*) and 2 cercopithecine/macaque herpesviruses (*B* and *C*). (*D–F*) Low and uneven coverage was found for the symbiont associated chlorella phycodnavirus (*D*), a coccolithophorid virus (*E*), and a fish iridovirus (*F*).

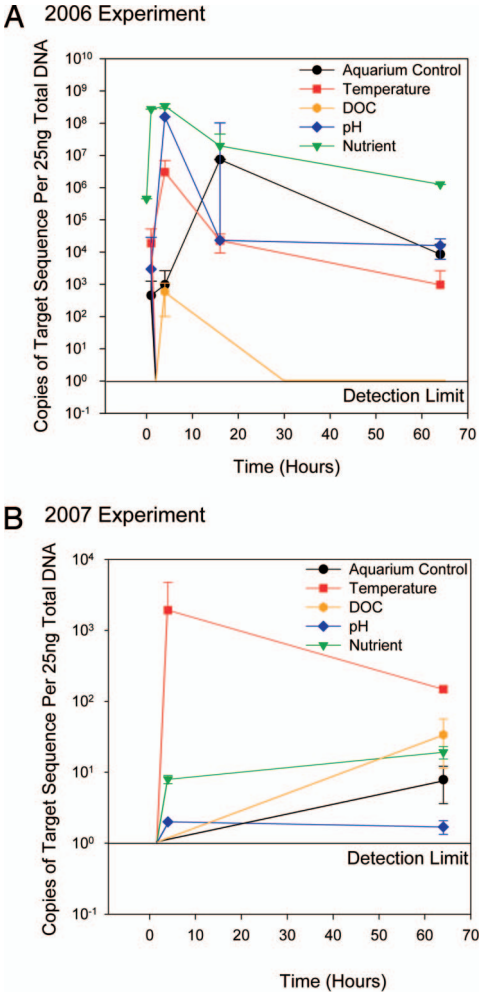

Fig. 3. Herpes-like viral sequence abundance across two 64-h experiments. (*A* and *B*) Real-time PCR on a putative herpes-like virus thymidylate synthetase gene was conducted on all temporal samples collected during the original metagenomic experiment (*A*) and samples from a second experiment conducted 1 year later (*B*). In the original experiment, coral specimens were exposed to thermal (5° C above ambient), dissolved organic carbon (25 mg·mL^{-1} glucose), nutrient (10 μM nitrate, nitrite, phosphate, and ammonium), and pH (1.7 units below ambient) stress for 64 hours. (*B*) In the second 2007 experiment, corals were acclimated to the tanks for 2 weeks before treatment with stressor, thermal stress was an elevation of seawater temperature 3°C, acidity stress was a 0.2 unit reduction of ambient pH, and samples were collected at 0, 4, and 64 h.

exception of the Nutrient (green triangles). The target sequence was also elevated in all of the 1-, 4-, 16-, and 64-h treated corals. The abundance of this target sequence typically peaked at 4 h after treatment exposure, and the maximum increase was ≈6 orders of magnitude. For example, the pH (blue diamonds) and Temperature (red triangles) stressors increased the abundance of this viral sequence from below the detection limit (≈10 copies) to >10^5 and 10^7 copies in only 4 h. Viral abundance was then reduced to 10^3 for the remainder of those experiments.

In the Aquarium Control sample (Fig. 3, black circles) large numbers (≈10^7) of this HLV specific target gene were detected after 16 h and demonstrated that even in the absence of applied exogenous abiotic stress, physical manipulation of the corals led to viral production. The viral sequence was detected in only 1 of the 5 DOC (orange hexagons) time points, corroborating the low abundance of sequences in the DOC metagenome. Both this low copy number in the DOC samples and the large copy number in the nutrient zero time point indicate that external herpesvirus contamination was not a confounding factor.

Second Experiment Conducted One Year Later Confirms that HLVs Are Commonly Associated with Stressed Corals. The possibility that the viruses discovered in the corals were unique to the original metagenomic experiment and/or the result of unnatural stress conditions was tested by conducting a second experiment 1 year later. This second repeat experiment used the same sampling scheme (Fig. S1) with minor modifications to reflect more likely environmental conditions: (*i*) corals were acclimated to the tanks for 2 weeks before treatment with stressor, (*ii*) thermal stress was an elevation of seawater temperature 3°C, (*iii*) acidity stress was a 0.2 unit reduction of ambient pH, and 4) nubbins were only collected at 0, 4, and 64 h. Identical primer sets were used to amplify the HLV thymidylate synthase sequence.

These analyses showed an increase in the herpes-like virus target sequence when stressors were applied to the corals (Fig. 3*B*). The target sequence was not detected at the zero hour time point in any of the tissue samples, but was found in the temperature (red squares), pH (blue diamonds), and nutrient (green triangles) samples at 4 and 64 h. The DOC exposed and aquarium control corals contained no detectable target sequence until 64 h.

Together, these results show that, although the temporal changes in HLV abundance were analogous in both experiments, the magnitude of this change was different. Compared with the 2006 metagenomic experiment, the total abundance of HLVs was lower in the second 2007 experiment. This may have resulted from the more environmentally relevant levels of stressors applied in the repeat experiment. However, environmental conditions were also milder during the second 2007 experiment with no extended rainy or windy periods before or during the experiment. In contrast, during the original 2006 experiment, precipitation along with significant runoff occurred. As previously mentioned, the abundance of the HLVs was found to be high in the original experiment's nutrient zero hour sample. This indicates that production of virus had already begun on the reef, perhaps as a result of degraded environmental conditions.

A two-way ANOVA conducted on the real-time data indicated significant effects of both treatment ($F = 9.80$, $P < 0.01$) and time ($F = 4.82$, $P < 0.01$), and a significant interaction effect ($F = 4.54$, $P < 0.01$). The combination of the two main effects and the interaction term explains >70% of the variance. These results indicated that the different treatments applied to corals stimulated herpes-like viral production and that the level of production varied significantly over the time course of the experiment. A significant interaction term suggested that the effects of both treatment and time on viral production were amplified multiplicatively. This implies that just studying each treatment individually over time or all treatments simultaneously over 1 time point would not have shown the same differences in production.

Additional Evidence for Herpes-Like Viruses in Corals and All Cnidarians. In addition to our datasets, transmission electron micrographs (TEM) and sequences from the *Nematostella* genome and hydra EST project provide further evidence that Cnidarians, not just corals, are hosts for herpes-like viruses. Corals contain large enveloped icosahedral virions morphologically similar to the *Herpesviridae* (28, 29). Herpesvirus essential genes are also present in Cnidarian genomes (28, 51, 52). Within the *Nematostella* draft genome, alkaline exonucleases, herpes major outer envelope proteins, herpes DNA primases, herpes ubiquitin-specific proteases as well a herpes surface glycoprotein and transcription activation factor were identified (Table S4). Also, several copies of a latent membrane protein were found suggesting the presence of endogenous or latent herpes-like viruses in *Nematostella*. Additionally, expressed sequence tags from *Hydra magnipapillata* contain herpes specific tegument proteins and herpes-associated proteases, suggesting that herpes-like viral transcripts were produced.

Herpes-like viral sequences were also recently identified in metagenomic libraries from *P. astreoides* and *D. strigosa* corals. In

the *P. astreoides* metagenome, most of the sequences with similarity to eukaryotic viruses were related to fish iridoviruses, but the second most abundant viral similarities were to *Herpesviridae* (30). In addition, >50% of the all viral sequence similarities (including phages and Archaeal viruses) from linker amplified shotgun libraries of healthy and bleached *D. strigosa* were to *Herpesviridae*, indicating that herpesviruses are commonly associated with corals and not a result of our methodology.

Caveats. The initial metagenomic experiment was designed both to address questions about the viral consortia present in corals and to determine whether this high-throughput metagenomic approach is amenable for empirical experiments. Therefore, treatments were designed to be harsh and as a result did not reflect realistic stressor levels. However, the second 2007 experiment was redesigned to represent more environmentally relevant levels of stress and used to confirm trends from the metagenomes.

In addition, equal amounts of DNA were combined from each replicate coral at each time point to generate individual stressor libraries that contained between 36 and 45 separately extracted viromes. This pooling approach provided a reduction in the variance, or normalizing shifts in the viral consortia over-time. The metagenomes therefore represent an underestimation of the true changes in the viral consortia. The individual temporal samples were then used for real-time PCR to verify identified trends.

The method used to collect the coral subsamples or nubbins required removal of the corals from the aquaria and repeated tissue damage to the coral finger. Corals from the no treatment aquarium control had elevated numbers of herpes-like viral sequences in both experiments. It was apparent from the real-time PCR that this method was disruptive enough to induce herpes-like viral particle production albeit at a reduced level compared with the other stressors. However, naturally occurring damage to corals results from predation, weathering events like hurricanes and earthquakes, and human activities, such as the aquarium coral and ornamental fish trade, recreational diving, and research (53). Our experimental design may have inadvertently mimicked fragmentation and small scale predation on corals. It is therefore likely that these activities and events are stressful enough to induce production of herpes-like viruses on the reef and may result in short or long term reductions in coral health that have until now gone unnoticed.

Last, the metagenomic DNA was amplified using multiple displacement amplification with Phi29 polymerase before sequencing, which could artificially inflate the occurrence of rare sequences, ssDNA, and small circular genomes, thus biasing the analysis (54, 55). However, multiple displacement amplification generally provides an even representation of genomes except at the ends (56) and, as evidenced by the elevation of ssDNA viruses in only the DOC sample, did not appear to bias these data.

Conclusions

This experiment used 6 empirically derived metagenomes to investigate shifts in previously unexplored viral consortia. The coral holobiont is a symbiotic community of animal host, zooxanthellar algae, and various other microbiota (57). This diversity of organisms is reflected in the viral consortia present within the tissues of corals, which included representatives of viruses that infect many protist and metazoan phyla (Fig. 1*B*), and viruses that infect Bacteria and Archaea (data not shown). Due to the predominance of herpes-like viral sequences in both our samples and *Nematostella*, an aposymbiotic anemome, we hypothesize that these distantly related herpesviruses infects the coral host.

Environmental and Physical Stressors Are Sources of Viral Production. The metagenomic and temporal experiments presented here demonstrate that exposure to stressors results in the production of a herpes-like virus or a consortium of herpes-like viruses in *P. compressa* corals. Thermal stress, eutrophication, and decreasing seawater pH have each been shown to disrupt coral health. Increases in sea surface temperature causes coral bleaching and increased coral disease incidence (58, 59). Nutrient addition exacerbates coral diseases, and reduced pH results in loss of corallite deposition (60, 61). This study demonstrates that, in addition to symbiont loss and bacterial and fungal disease (62), temperature and nutrient elevation and pH reduction result in increased HLV production.

A hypothesis that was not directly tested is that the duration of each stressor maybe critical to the overall effect on viral production. Treatments were only 64 h in length, but stress events may last significantly longer on the reef. A reduction in the viral abundance after 4 h in both experiments may suggest that corals can withstand these short stressors, but prolonged stress, such as sustained seawater temperature elevation, may overwhelm a coral's ability to fight off infection.

It is currently unclear how these viruses affect coral physiology or ecology. We hypothesize that viral production can (*i*) directly cause down stream changes in physiology such as a reduced innate immunity response, secondary opportunist infection by microbes, symbiont expulsion, cell detachment, and apoptosis, or (*ii*) negatively combine with other stressor induced changes.

Herpesviruses, Human Disease, and the Evolution of Metazoans. Herpesviruses are characterized by several morphological and genomic commonalities, including large enveloped icosahedral virions, long linear double stranded DNA genomes, and similar life cycle pathways (63). Although herpesviruses were once considered exclusively vertebrate diseases, members of the ostreid *Herpesviridae* are known to infect and kill invertebrates, specifically industrially cultured bivalves (64). The genomes of these shellfish-infecting herpesviruses contain few gene sequences in common with their vertebrate counterparts. Genome examination has demonstrated that herpesviruses can contain upwards of 70% unique genes (65). Across all of the genomes of *Herpesviridae* there is only a single subunit of a terminase shared in common (66). These data suggest that *Herpesviridae* is not a phylogentically related group of viruses, but perhaps consists of 2 phenetic Families, one related to vertebrate disease and the other to invertebrate disease.

Our coral metagenomes contain more sequences similar to the α-herpesviruses (including several human associated herpesviruses), which infect predominantly mammals and often exhibit broad host range (37). The data presented here support the hypothesis that herpes-like viruses are common in Cnidarians and could perhaps represent distant relatives of the vertebrate lineage of *Herpesviridae*. Our studies may also provide insight into the evolution of these viruses. Herpesviruses typically infect nervous tissue, and it is tempting to suggest that the herpes-like viruses in Cnidarians may have ancient origins, because Cnidarians are the first metazoans to develop rudimentary nervous systems (52).

This was the first experiment to use massively parallel pyrosequencing for empirical experiments, demonstrating that this high-throughput approach can be used as a first step to identify novel viruses in virtually any system. Yet caution should be taken when designing empirical metagenomics. Our statistical analysis on the real-time PCR suggests static analysis of metagenomes may fail to identify critical changes between and among viral consortia.

Overall, we have demonstrated that herpes-like viruses are commonly associated with corals and perhaps all Cnidarians and are produced as a result of changes in ambient water conditions and physical disruption. It is not yet clear how these viruses may affect the physiology or resilience of their hosts, but these data provide a critical foundation for future studies on eukaryotic viral disease ecology of corals.

Materials and Methods

Virome Generation. Virome DNA from each time point and treatment was isolated and extracted to generate twenty-five temporal samples that were

subsampled, amplified, and then pooled by treatment for metagenomic library construction (see Fig. S1).

Virome Purity Verifications. To verify that the viral libraries contained no human contaminating DNA, sequences were compared with the human genome using BLASTn and a minimum 200 score as measured by the formula (0.267 × raw score-ln(0.041))/ln (2)). Raw scores were the sums of the alignment pairwise scores. Across all 6 viromes low numbers of sequences (<0.8%) were assigned ''human'' indicting that human contamination was not an issue.

Real-Time PCR. Real-time PCR on the putative herpes-virus target sequence was conducted using SYBR GreenER qPCR SuperMix from Invitrogen according the to the manufactures' recommendations with exactly 25 ng of total viral DNA. Primers used were: forward, 5′-AAAATAAGATTGGGAGATCTAGGC-3′ and reverse, 5′-TGCCATTTTAGGTAAATCAGAAAC-3′. Standards were made from purified target and standard curves were generated using 0.5, 0.05, 0.005, and 0.0005 ng of the target (GenBank accession no. EU660955). A two-way ANOVA (time and treatment) was conducted using Minitab Version 15 (State College, PA). Both parametric and non-parametric tests provided similar results, and the residuals were randomly distributed with a mean of 0, justifying the assumptions.

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Supporting Information

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SI Methods

Virome Generation. Metagenomic viral DNA extractions: Viral particles were isolated using cesium chloride (CsCl) gradient centrifugation. Coral slurries were centrifuged at 3,000 rpm for 15 min to remove coral debris, and the supernatant was placed in a new 50 mL conical tube. CsCl gradients were made with FASW, and loaded in the following densities: 1.7, 1.5, and 1.35 $mg{\cdot}mL^{-1}$, respectively. To each gradient, ≈9 mL of sample supernatant was added. Gradients were then centrifuged at 82,000 × *g* for 2 hours in a Beckman Ultracentrifuge at 4°C. The 1.5–1.35 $mg{\cdot}mL^{-1}$ fraction was removed with an 18-gauge needle on a sterile syringe and run through an additional gradient to ensure that all bacteria and debris were removed. A subsample of the resulting fraction was analyzed for the presence of contaminating eukaryotic and microbial cells using Sybr Gold (Invitrogen) staining and epifluorescence microscopy as described in ref. 1. The viral fraction was then DNase I treated to remove any residual free DNA. Viral DNA was extracted using formamide and CTAB, as described in ref. 1. To verify the absence of contaminating eukaryotic and microbial DNA 16S and 18S PCR was conducted on all samples before sequencing as described in ref. 2. No bands were detected.

Virome DNA from each time point and treatment was isolated and extracted to generate twenty-five temporal samples, which were subsampled, amplified, and then pooled by treatment for metagenomic library construction (Fig. S1). Once purified, 1 ng of viral genomic DNA from all samples underwent amplification, using GenomiPhi from GE LifeSciences (Quebec, Canada) and repurification with a DNeasy Blood and Tissue Kit from QIAGEN (Valencia, CA). Each of the metagenomic libraries included equal amounts of the amplified DNA from the 1, 4, 16, and 64 h isolates of each corresponding treatment, except the reference sample time zero, which was a pool of all of the time 0 temporal samples (Fig. S1). Approximately 5 μg of total genomic DNA for each library was sent to 454 Life Sciences (Branford, CT) for pyrosequencing using GS20 technology (Fig. S1).

Each of the reads was parsed, stored, assigned a number, and archived at the San Diego State Center for Universal Microbial Sequencing (http://scums.sdsu.edu). Each viral metagenome can be accessed through this website under the accession nos. 4440374.3, 4440375.3, 4440370.3, 4440371.3, 4440377.3, 4440376.3, but are also found at National Center for Biotechnology Information as genome projects IDs: 28427, 28429, 28431, 28433, 28435, and 28437.

Repeat stressor experiment: All parameters were identical to the initial experiment except that in the Temperature treatment, water was increased to 3°C above ambient instead of 5° C. Also, in the ocean acidity stressor, seawater pH was reduced to 7.8 to reflect a more likely environmental change. Salinity, temperature and pH were measured at every sampling.

Herpes-like thymidylate synthase contig assembly. Sequences from each metagenome that were annotated as herpes-like were assembled using SeqMan from DNASTAR Inc. (Madison, WI) using 99% similarity, 35 base pair overlaps, and a minimum sequence read length of 80 base pairs. Contigs were generated and consensus viral sequences were identified using PSI-BLAST to the nonredundant database at National Center for Biotechnology Information. Primer sets were generated and used first for conventional PCR and then real-time PCR, cloning, and Sanger sequencing.

PCR and putative herpes-like thymidylate synthase gene sequence cloning. For the putative herpes-like virus thymidylate synthase gene, PCR was carried out in 50 μL of standard reactions containing 200 nM primers and 25 ng of total viral DNA. Touch down thermocycling was conducted using a 3 min 95°C hot start and 30 cycles of the following: 95°C for 1 min, 60°C (−0.5°C) for 30 seconds, and 72°C for 1 min. A 10-min extension at 72°C completed the PCR. Samples were run on a 1 or 1.5% agarose gel. PCR reactions were purified using a PCR AccuPrep Kit from BioNeer and cloned using a TopoTA Kit from Invitrogen. Sequencing was conducted at the CSU-PERB Microchemical Core Facility. Sequences were trimmed by hand and homology identified using PSI-BLAST to the nonredundant database at National Center for Biotechnology Information.

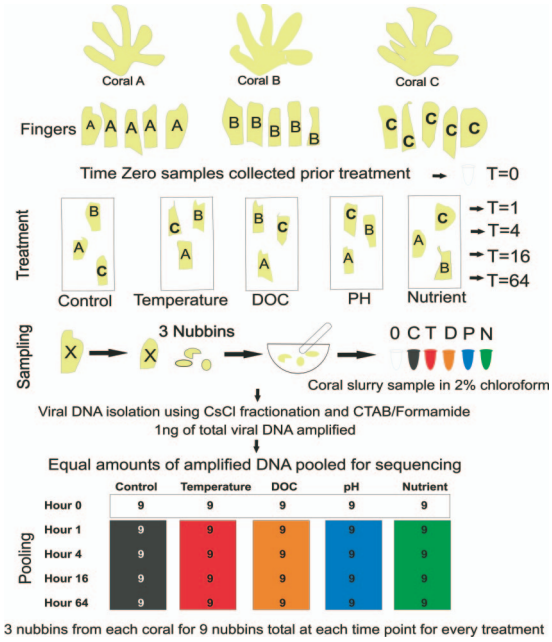

Fig. S1. Coral collections: *Porites compressa* specimens were collected on February 22, 2006 (metagenomic experiment) and February 27, 2007 (repeated stressor experiment) directly off Reef Point (≈21°.25′ N, 157°47 W) on Coconut Island, Kane'ohe Bay, O'ahu, Hawai'i. Samples were collected 25 m off shore, at 1-meter depth and 5 meters apart. Coral heads of ≈20 cm in diameter were removed with hammer and chisel at the base of the colony to avoid polyp damage. Specimens were placed in a 3 L seawater cured bucket, carried to shore, and immediately transferred to large open outdoor aquaria that contained continuous and rapidly flowing seawater.

Environmental stressor experiments for viromes: The 3 coral heads (*A*–*C*) were each broken into 5 ''fingers'' of approximately equal size (≈30–40 grams), and 3 parts or ''nubbins'' along each finger were sampled before placement in tanks creating the Time Zero reference samples (Fig. S1). One coral finger from each head was then placed in a glass aquarium (12.5 × 5 × 8), containing treated sand filtered seawater. The Aquarium Control sample received no treatment but similar manipulations (placement in tank, removal from tank etc…). Water for the controls was 5 L of sand-filtered seawater from the HIMB aquaria facility. Temperature treatment was a 1-time increase (not ramped) of the tank seawater temperature from local ambient, 24°C, to a maximum of 30°C. Nutrient treatment consisted of a 10 μM excess of: nitrate ($Ca(NO_3)_2$), nitrite ($NaNO_2$), ammonium (NH_4Cl), and phosphate (KH_2PO_4). Dissolved organic carbon loading was addition of 25 mg·L^{-1} glucose, and pH treatment was a reduction of ambient pH from 8.0 to 6.8 using hydrochloric acid and bicarbonate. Ambient conditions included lower than average air temperatures and high precipitation rates. Aquarium treatment water/stressor was replenished every 24 h.

At each time point (1, 4, 16, and 64 h) all 3 fingers were sequentially removed from each tank and placed on sterile aluminum foil. Then 3 distinct, 1–2 gram, ''nubbins'' (tissue and skeleton) were cut away with a sterile razor blade, and the finger was immediately returned to the tank. This resulted in 9 different tissue samples for each treatment at each time point. Each nubbin was rinsed with filtered (0.02 μm) autoclaved sea water (FASW), placed in a precleaned mortar and pestle in the presence of ≈10 mL FASW, crushed into a slurry, and rinsed into a 50 mL of BD Falcon tube with an additional 10 mL of FASW. Approximately 0.2 volumes of molecular biology grade chloroform was added to the slurry to kill any microbes or eukaryotes present, mixed vigorously for several minutes and placed at 4°C until shipping. This was repeated for every coral (*A*–*C*) to generate a total pool of 9 nubbins at each time point (1, 4, 16, 64 h) for the aquarium control, temperature, DOC, pH, and nutrient treatments. Samples were stored at 4°C and shipped on ice to San Diego State University for processing and pooling before pyrosequencing. Metagenomic libraries were generated by pooling DNA in equal amounts from each of the viromes (each of the different 9 nubbin pools). Real-time PCR was conducted on each of the 3 coral sets (*A*–*C*) at each time point for every treatment (5 treatments, 5 time points) for a total of 25 samples in triplicate. All samples were then shipped on ice to San Diego State University for processing.

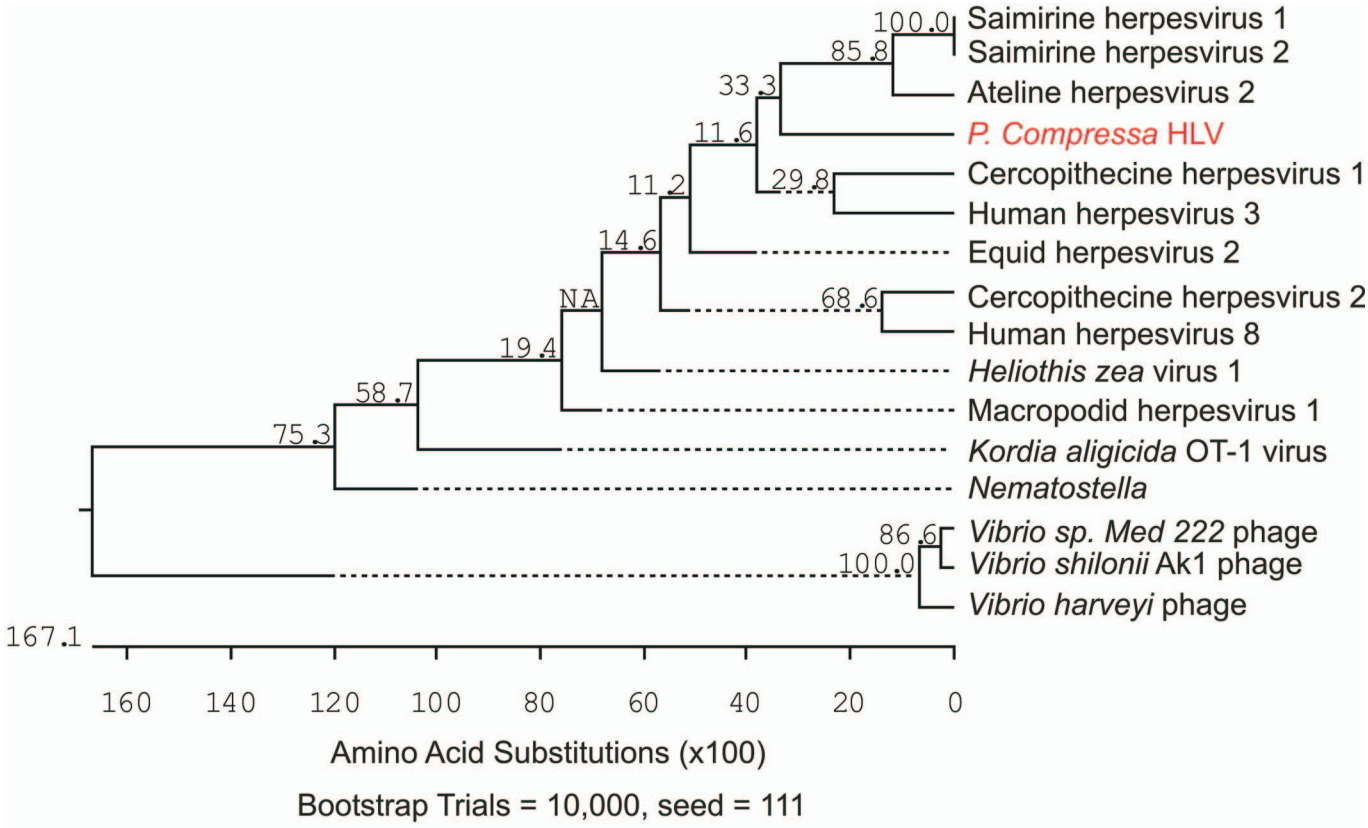

Fig. S2. Coral associated herpes-like viral sequence used for real-time PCR. Sequences from several viral, bacterial, and anemone thymidylate synthase proteins were aligned to the putative Coral HLV sequence using CLUSTAL W and MegAlign from DNASTAR. Blosum 30, PAM 350, and Gonnot 250 matrixes all produced the same tree. Bootstrap analysis was performed using 10,000 iterations.

Table S1. Metagenome characteristics and similarity statistics when compared against the non-redundant ($e < 10^{-4}$) database at NCBI using BLASTn. Each sequence with a similarity in the NR was given a taxonomic assignment (viral, bacterial, eukaryotic) based on its best (smallest e value) similarity

Sample	Reads	Approx. read length	Known, %	GC content, %	Viral, %	Bacterial, %	Eukaryotic, %
Time zero	39,270	101.32	2.12	42.25	1.87	64.14	33.14
Control	39,340	103.7	5.21	46.48	2.89	90.18	6.05
Temperature	39,036	113.38	2.01	48.27	0.98	58.09	40.05
DOC	35,680	102.18	1.88	42.49	8.71	71.24	19.55
pH	50,368	104.73	1.57	43	3.37	68.67	27.44
Nutrient	34,433	107.18	1.83	45.1	6.92	76.82	15.32

Table S2. Coral-associated viral metagenomes were compared for similarity to four previously isolated marine water viromes

Sample	Kingman Reef	Christmas Reef	Palmyra Reef	Tabuaren Reef
Time zero	10.53	10.88	11.10	6.52
Control	2.49	3.65	5.57	1.13
Temperature	29.07	20.12	38.14	25.44
DOC	1.58	2.46	1.74	0.77
pH	11.91	9.63	16.29	9.59
Nutrient	17.41	11.81	23.04	14.27

Percentage similarities between each coral library and reef water viral library were calculated using BLASTn ($e < 10^{-4}$).

Table S3. Examples of combined viral metagenome coverage to 2,020 fully sequenced viral genomes from NCBI

Virus	Accession no.	Genome size, kb	Family	Similarities	Coverage
A. polyphaga mimivirus	NC_006450	1181.4	Mimivirus	952	0.09
A. tigrinum virus	NC_005832	106.33	Iridoviridae	64	0.07
A. moorei entomopoxvirus	NC_002520	232.4	Poxviridae	333	0.16
Bovine herpesvirus 1	NC_001847	135.3	Herpesviridae	1071	**0.87**
Bovine herpesvirus 5	NC_005261	137.82	Herpesviridae	360	0.29
Canarypox virus	NC_005309	359.85	Poxviridae	168	0.05
Cercopithecine herpesvirus 1	NC_004812	156.8	Herpesviridae	666	**0.47**
Cercopithecine herpesvirus 16	NC_007653	156.5	Herpesviridae	976	**0.69**
Cercopithecine herpesvirus 2	NC_006560	150.72	Herpesviridae	1282	**0.94**
E. siliculosus virus	NC_002687	335.59	Phycodnaviridae	174	0.06
E. huxleyi virus 86	NC_007346	407.34	Phycodnaviridae	725	0.20
Equid herpesvirus 2	NC_001650	184.43	Herpesviridae	176	0.10
Human herpesvirus 1	NC_001806	152.26	Herpesviridae	216	0.16
Human herpesvirus 2	NC_001798	154.746	Herpesviridae	283	0.20
M. sanguinipes entomopoxvirus	NC_001993	236.12	Poxviridae	169	0.08
P. bursaria Chlorella virus 1	NC_000852	330.74	Phycodnaviridae	414	0.14
Shrimp spot syndrome virus	NC_003225	305.11	Nimaviridae	125	0.05
Singapore grouper iridovirus	NC_006549	140.13	Iridoviridae	169	0.13
Suid herpesvirus 1	NC_006151	143.46	Herpesviridae	862	**0.66**
Tupaia herpesvirus	NC_002794	195.86	Herpesviridae	238	0.13

All coral-associated viral metegenomes were combined and the number of sequence similarities ($e < 10^{-6}$) to each genome was calculated. Frequency tables were generated for each 2,500-bp bin across each reference genome. First, the number of synonymous nucleotides between the metagenome and the reference genome was counted. Coverage was then calculated by dividing this number by the total length of reference the genome. Any genome with coverage equal to or greater than 0.05 is listed. Coverage values in bold denotes the 5 highest values.

Table S4. Herpes-like viral sequences in the reference genomes of two Cnidarians

Nematostella Draft	Hydra ESTs
5507928	74131562
5500715	74132680
5503168	68410998
5522046	
5497885	
5517497	
5512689	
5521542	
5498193	
5515317	
5515317	
XP_001626845	
EDO34745	
XP_001641920	
EDO49857	
XP_001636448	
EDO44385	
XP_001629245	
XP_001627817	
XP_001626452	
XP_001624299	
XP_001621368	
XP_001617849	
EDO25749	
EDO29268	
EDO32199	
EDO34352	
EDO35717	
EDO37182	