

# 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  $0.8\text{--}1 \times 10^9$  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  $\approx 2\text{--}4$  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.

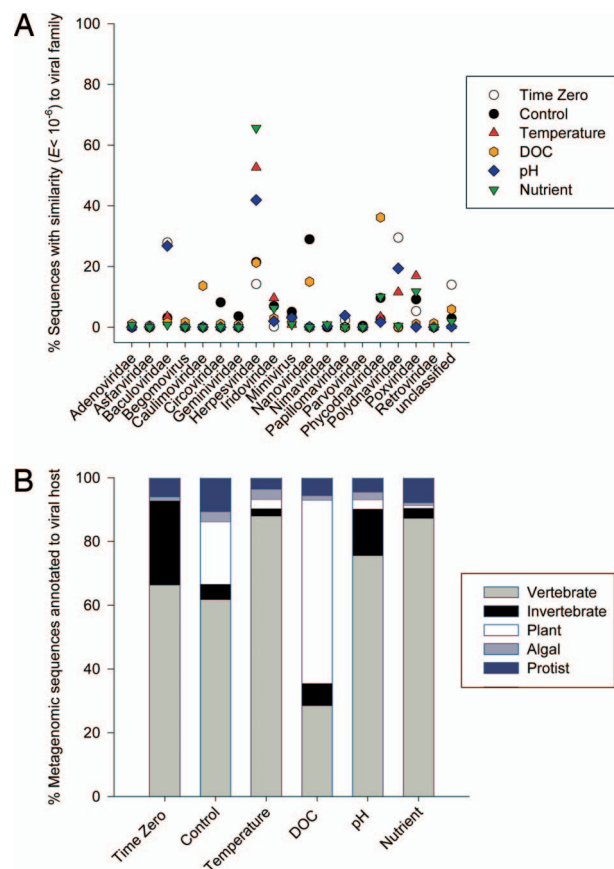
Coral specimens were exposed to thermal ( $5^{\circ}\text{C}$  above ambient), dissolved organic carbon ( $25\text{ mg}\cdot\text{mL}^{-1}$  glucose), nutrient ( $10\ \mu\text{M}$  nitrate, nitrite, phosphate, and ammonium), and pH (1.7 units below ambient) stress for 64 hours [supporting information (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  $1\ \mu\text{g}$  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  $\approx 100$  viral families described thus far (37), 19 were found



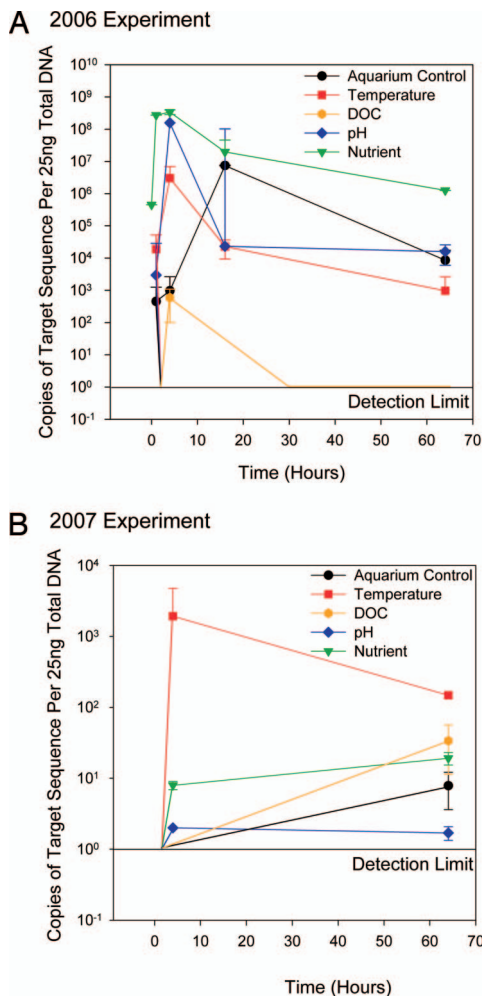
**Fig. 1.** A) Percentages of best similarities (tBLASTx,  $e \leq 10^{-6}$ ) to 19 families of viruses in each individual coral-associated viral metagenome: Time Zero (open circles), Aquarium Control (filled circles), Temperature (red triangles), pH (blue diamonds), Nutrient (green arrows), and DOC (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. 1A). 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 on 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. 1B). 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-







**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<sup>-1</sup> 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<sup>0</sup> copies) to >10<sup>5</sup> and 10<sup>7</sup> copies in only 4 h. Viral abundance was then reduced to 10<sup>3</sup> for the remainder of those experiments.

In the Aquarium Control sample (Fig. 3, black circles) large numbers (≈10<sup>7</sup>) 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 (iv) nubbins were only collected at 0, 4, and 64 h. Identical primer sets were used to amplify the HLV thymidylate synthetase sequence.

These analyses showed an increase in the herpes-like virus target sequence when stressors were applied to the corals (Fig. 3B). 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 as 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. 1B), 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 anemone, 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 phylogenetically 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  $\alpha$ -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  $(0.267 \times \text{raw score} - \ln(0.041)) / \ln(2)$ . Raw scores were the sums of the alignment pairwise scores. Across all 6 viromes low numbers of sequences (<0.8%) were assigned "human" indicating 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 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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