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Temperature alters algicidal activity of DNA and RNA viruses infecting *Chaetoceros tenuissimus*

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ABSTRACT: Viral infection is considered a significant factor affecting the dynamics of diatoms in marine environments. Two different diatom viruses — the single-stranded DNA virus CtenDNAV and the single-stranded RNA virus CtenRNAV, that both infect and lyse the marine planktonic diatom *Chaetoceros tenuissimus* Meunier — have been isolated from western Japan. To understand the ecological relationship explaining how they share the same host species in nature and how the host population survives, basic host growth and physiology of viral infections should be examined. We evaluated the relationship between host growth rates and viral infections with respect to water temperature within the range of 10 to 30°C. The maximum growth rates of 2 different strains of *C. tenuissimus* were 3.4 to 3.5 divisions d^{-1}, which were obtained at 25 to 30°C. The viral infection tests showed that the decline in the host population due to CtenDNAV was faster at higher water temperatures. The susceptibilities of the host strains to CtenRNAV (but not to CtenDNAV) were diverse, and varied with water temperature. The results indicate that successful CtenDNAV proliferation occurs during seasons with higher water temperature that are more suitable for host growth. However, CtenRNAV proliferation does not necessarily correlate with temperature-dependent host growth. The host populations grow in the water column during the course of a year, and the different temperature-dependent viral proliferations allow the 2 viruses to coexist in a narrow spatial region. Additionally, the viral infections seem to be accelerated during the host stationary growth phase, but not during the logarithmic growth phase, which might contribute to host population survival in nature.

KEY WORDS: Diatom · CtenDNAV · CtenRNAV · Virus · Niche partitioning

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INTRODUCTION

In response to reports of a high abundance of virus-like particles in natural waters, aquatic viruses have been intensively examined (Bergh et al. 1989, Wommack & Colwell 2000). Currently, viruses are regarded as one of the major biological factors that regulate carbon cycling and microbial biomass (Suttle 2005, Brussaard et al. 2008). Environmental factors appear to be significant in determining the fate of a viral infection in its host organism because they are critical factors affecting metabolic activity and biological processes. In bloom-forming microalgae and their virus systems, diverse environmental factors considerably affect their relationships (Brussaard 2004); among them, water temperature is one of the most significant factors. For example, *Heterosigma akashiwo* (Raphidophyceae) is sensitive to its large double-stranded DNA (dsDNA) virus HaV at 25°C, but the host can resist viral infection at higher temperatures (Nagasaki & Yamaguchi 1998). The dynamics of a bloom of the harmful algal species *Phaeo-*

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cystis globosa (Prymnesiophyceae) are affected by several distinct groups of its infectious viruses, and their interactions and potential dominance are strongly controlled by water temperature (Baudoux & Brussaard 2005). Therefore, the effects of water temperature should be investigated for essential understanding of host–virus relationships.

Diatoms are unicellular, eukaryotic algae found throughout the world's oceans and freshwater systems, and they account for a large part of marine primary production — up to 35% in oligotrophic oceans and 75% in nutrient-rich systems (Nelson et al. 1995). As major primary producers in nutrient-rich systems, diatoms are vital in supporting large-scale coastal fisheries. Several field studies have reported the potential importance of viral influences on diatom dynamics in marine environments (Bettarel et al. 2005, Tomaru et al. 2011a), as well as physical and chemical factors (Sarthou et al. 2005). To date, 14 different diatom viruses have been isolated and characterised (Tomaru et al. 2012, 2013, Kimura & Tomaru 2013). Recently, 2 viruses with different genome types (single-stranded DNA [ssDNA] and ssRNA) that share the same diatom host have been reported. CtenDNAV is an ssDNA virus that infects the bloom-forming marine diatom *Chaetoceros tenuissimus* Meunier strains 2-6 and 2-10 (Tomaru et al. 2011b). CtenRNAV is an ssRNA virus that causes the lysis of *C. tenuissimus* strain 2-10 (Shirai et al. 2008), but not strain 2-6. These 2 viruses and their host species have been isolated from western Japan and are considered important factors affecting host dynamics (Tomaru et al. 2011a). Consequently, we are interested in understanding how the 2 viruses share the same host species in the same region. To understand the ecology of *C. tenuissimus* and its viruses, and the survival and coexistence of the 2 viruses, we primarily examined the growth rates of 2 different *C. tenuissimus* strains and CtenDNAV and CtenRNAV infections with respect to water temperature.

Furthermore, as well as environmental factors, host growth conditions are important factors affecting host and virus systems. For example, hosts *Chlorella variabilis* (Trebouxiophyceae) (Van Etten et al. 1983), *Phaeocystis pouchetii* (Prymnesiophyceae) (Bratbak et al. 1998) and *Heterocapsa circularisquama* (Dinophyceae) (Nagasaki et al. 2003) show accelerated viral production during the host's vigorous growth phase, but this decreases in the stationary phase. In contrast, diatom populations in logarithmic growth phases do not decline due to viral infection, but they do decline in stationary phases (Shirai et al. 2008, Tomaru et al. 2011b). The difference might be a clue to understanding diatom population survival. Therefore, in the present study, we aimed to explore this phenomenon using a semi-continuous culture method.

MATERIALS AND METHODS

Algal cultures and growth conditions

The axenic, clonal, algal strains used in this study, *Chaetoceros tenuissimus* strains 2-6 (Tomaru et al. 2011b) and 2-10 (Shirai et al. 2008), were isolated from surface water in Hiroshima Bay, Japan on 9 July 2002 and from Harima-Nada, Seto Inland Sea, Japan on 10 August 2002, respectively. The strains 2-6 and 2-10 were used previously for isolation of CtenDNAV (Tomaru et al. 2011b) and CtenRNAV (Shirai et al. 2008), respectively. Algal cultures were primarily maintained in SWM-3 medium (ca. 30 PSU) enriched with 2 nM Na_2SeO_3 (Imai et al. 1996), under a 12:12 h light:dark cycle of approximately 110–150 µmol of photons m^{-2} s^{-1} using cool white fluorescent illumination at 15°C.

Viral inocula

Exponentially growing cultures of *C. tenuissimus* strains 2-6 and 2-10 were inoculated with CtenDNAV and CtenRNAV, respectively, and were incubated for 7 d. The lysates were passed through a 0.2 µm polycarbonate membrane filter (Nuclepore) to remove cellular debris and were stored at 4°C in the dark. Filtered lysates were used as the inocula for the experiments in this study.

Effects of temperature and salinity on host diatom growth

The culture experiments were conducted at 5 temperatures (10, 15, 20, 25, and 30°C) in combination with 6 salinities (10, 15, 20, 25, 30, and 35 PSU) in temperature gradient growth chambers (TG-100-AD, Nippon Medical and Chemical Instruments) under the light conditions stated above. The salinity of seawater was adjusted from 30 to 10 PSU by dilution with distilled water. A salinity of 35 PSU was obtained by concentrating the natural seawater in a drying oven at 50°C. Seawater was enriched with the SWM-3 medium, as mentioned above, after the salinity adjustments.

Cells were pre-conditioned to the experimental conditions through stepwise transfer of stock cultures to each temperature and salinity regime. If transferred cells grew at the experimental regime, then the culture was conditioned at that regime for at least 2 wk; if cells did not grow, the growth experiment was not carried out, and the growth rate at that temperature and salinity regime was regarded as zero. Although we carefully conducted the stepwise transfer of the host cultures, apparent growth was unfortunately not observed at the temperature and salinity regimes of 10°C and 10 PSU for host strain 2-10, nor at 10°C and 10 to 15 PSU for strain 2-6. Acclimated stock cultures were inoculated into triplicate polypropylene-capped test tubes (13 × 150 mm) for each experimental regime. Inoculum size was adjusted to <1:1000 v/v of pre-cultures.

Growth rates in triplicate cultures were determined by measuring the *in vivo* chlorophyll *a* fluorescence every 24 h using a Turner Designs Model 10-100 R Fluorometer. Growth rates (μ; divisions d^{-1}) were calculated in triplicate using data from the exponential portion of the growth curve by least squares regressions of the natural logarithm of fluorescence on day number, as previously described (Yamaguchi & Honjo 1989). The mean growth rate was calculated using the 3 independent estimates of μ. A fluorescence decline from the initial value was considered a zero growth rate. A 2-way analysis of variance (ANOVA) was conducted to estimate the effects of temperature, salinity, and their interaction on the growth rates. Based on the results obtained from the statistical analyses, we determined cubic polynomial equations of the form:

$$\mu = b_{00} + b_{10}T + b_{20}T^2 + b_{30}T^3 + b_{01}S + b_{02}S^2 + b_{03}S^3 + b_{11}TS + b_{12}TS^2 + b_{21}T^2S$$

where μ = growth rate, T = temperature (°C), S = salinity (PSU), and b_{nm} = regression coefficients, in which the subscripts representing the multipliers of the variable T^nS^m were fitted by the multiple regression analysis. For validation of the accuracy of the regression analytical model, we examined the correlation coefficient and the significance of difference between observed and calculated values. Goodness of fit was quantified using Akaike's information criterion.

Cross-reactivity tests under various water temperatures

The cross-reactivity assays between the host and 2 virus clones were tested by adding 5% (v/v) aliquots of fresh viral suspensions to duplicate cultures of 8 different exponentially growing *C. tenuissimus* clonal strains (see Fig. 2). The strain AG07-C03 was isolated from surface water in Ago Bay, Japan on 4 July 2007. The *C. tenuissimus* strains Cten-01, -07, -11, -13, and -24 were isolated from sediment samples collected in Ariake Sound, Kyushu, Japan in December 2009. A 0.5 g sediment sample was inoculated into 25 ml of SWM-3 medium and cultured under the light conditions mentioned above at 20°C. After 3 d, *C. tenuissimus* cells were isolated from the culture. Species identification of the isolated *C. tenuissimus* was conducted based on morphological observations under light microscopy and real-time PCR assays, as described previously (Toyoda et al. 2010). The virus-inoculated host strains were cultured under the conditions given above at 10, 15, 20, 25, and 30°C.

Growth, cell condition, and evidence of lysis in each algal culture on the bottom of the culture vessel were monitored by optical microscopy (Nikon Ti) and were compared with control cultures inoculated with the SWM-3 medium. Algal lysis was scored when lysed cells were observed at the bottom of culture vessels. Lysis was scored when at least >50% of the cells were lysed; partial lysis was scored when some lysis occurred, but >50% of the cells survived in the culture vessel. Lysed cells were visually distinguished from live cells based on pigment colour contrasts, i.e. the dead cells are pale in colour. Cultures not lysed at 16 d post-inoculation (dpi) were scored as unsuitable hosts for the viral pathogen. The observations were completed and the degree of lysis was scored in the virus-inoculated culture when the control cultures degraded (within 16 dpi).

Effects of water temperatures on viral proliferation

A late exponentially growing culture of *C. tenuissimus* (150 ml), with cell concentration between 5.0×10^5 and 2.0×10^6 cells ml^{-1}, was inoculated with its infectious viruses, CtenDNAV or CtenRNAV, at multiplicities of infection (MOI) between 10^0 and 10^2 (see Table 1). A *C. tenuissimus* culture inoculated with an autoclaved culture of SWM-3 served as the control. An aliquot of the cell suspension was sampled from each culture during experimental periods every day, and the number of host cells, viral infectious units, and occurrence of viral genome replication and accumulation in the host cells were estimated. This experiment was a single trial. Cell counts were carried out using a Fuchs-Rosenthal Haemocy-

tometer by optical microscopy (Nikon Ti), without fixation of the samples. The number of viral infectious units was determined using the extinction dilution method (Suttle 1993). Briefly, the samples used for estimations of the viral infectious units were passed through 0.8 µm polycarbonate membrane filters (Nuclepore) to remove cellular debris. These filtrates were diluted with the SWM3 medium in a series of 10-fold dilution steps. Aliquots (100 µl) of each dilution were added to 8 wells in cell-culture plates with 96 flat-bottom wells and were mixed with 150 µl of an exponentially growing culture of host algae. The cell culture plates were incubated at 15°C under a 12:12 h light:dark cycle of 130–150 µmol photons m^{-2} s^{-1} with cool white fluorescent illumination, and cultures were monitored by optical microscopy (Nikon Ti) over 14 d for culture lysis. The culture lysis due to a virus infection was usually observed as an almost complete degradation of the host cells in a well. We calculated virus abundance from the number of wells in which algal lysis occurred using a BASIC program, as described previously (Nishihara et al. 1986). The burst size was calculated based on the amount of increased infectious units per decreased host cell number in the same period. The cells in 2 ml of the sample were pelleted and stored at −80°C until Northern blot or Southern dot-blot analysis. DNA and RNA from the pelleted cells were extracted with DNeasy (Qiagen) and RNeasyPlus Kits (Qiagen), respectively. Viral genome replication and accumulation in the host cells were evaluated using the methods described in the Supplement at www.int-res.com/articles/suppl/a073p171_supp.pdf.

Semi-continuous culture

A semi-continuous culture experiment was designed to estimate the percentage of viral-induced lysed cells in a host population growing at 2 divisions d^{-1}. *C. tenuissimus* strain 2-10 was pipetted into a flask and incubated for 3 d at 15°C under the light conditions described above, and 75% of the culture was replaced with fresh SWM-3 medium every 24 h. After 3 d of the semi-continuous culture, 2 fresh virus-added cultures (CtenDNAV and CtenRNAV) and 1 control were prepared. In the virus-added conditions, CtenDNAV and CtenRNAV suspensions were pipetted into the culture at MOI of 0.03 and 4.9, respectively. A culture inoculated with an autoclaved culture of SWM-3 served as the control. This experiment was a single trial. The daily cell-decreasing rate of the host population, during the semi-continuous culture period for the CtenRNAV experiment, was estimated by linear regression analysis. The slope of the regression indicates the percentage of the cells that do not contribute to the host population growth, i.e. virus infection-permissive cells.

One day after viral inoculation, 2 replicates of the 75% dilution cultures were prepared for each experimental condition: CtenRNAV, CtenDNAV, and control cultures. One set of the cultures was used for the semi-continuous culture experiment, and the other set was used for a batch culture experiment, i.e. no dilutions. They were cultured at 15°C under the light conditions described above. The culture volume remaining after the daily dilution in the semi-continuous cultures was used to estimate the number of host cells, viral infectious units, and occurrence of viral genome replication and accumulation in the host cells, as described above. For the batch culture experiments, an aliquot of the cell suspension was sampled from each culture every day. The number of host cells and viral infectious units in the batch cultures were measured every 1 d and every 2 d, respectively.

RESULTS

Effects of temperature and salinity on growth of *Chaetoceros tenuissimus* strains

Chaetoceros tenuissimus strains 2-6 and 2-10 grew at water temperatures of 10 to 30°C and showed a tolerance to a wide range of salinities, from 15 to 35 PSU (Fig. 1). The strains did not grow under combinations of lower water temperature and lower salinity, <15°C and 10 PSU for strain 2-6 and 10°C and 10 PSU for strain 2-10 (Fig. 1). The maximum growth rates of *C. tenuissimus* strains were 3.4 divisions d^{-1} at 30°C and 20 PSU for strain 2-6 and 3.5 divisions d^{-1} at 25°C and 20 PSU for strain 2-10. Our results obtained from statistical analyses showed significant effects of temperature ($p < 0.001$), salinity ($p < 0.001$), and temperature–salinity interaction ($p < 0.001$) on growth rates of *C. tenuissimus* strains. In the ANOVA results, the sum of squares of temperature for growth rates of *C. tenuissimus* strains were 6-fold higher than those of salinity and temperature–salinity interaction; thereby, temperature is the most important factor influencing growth of *C. tenuissimus* strains. Based on the results, the cubic equations for each strain were determined as follows:

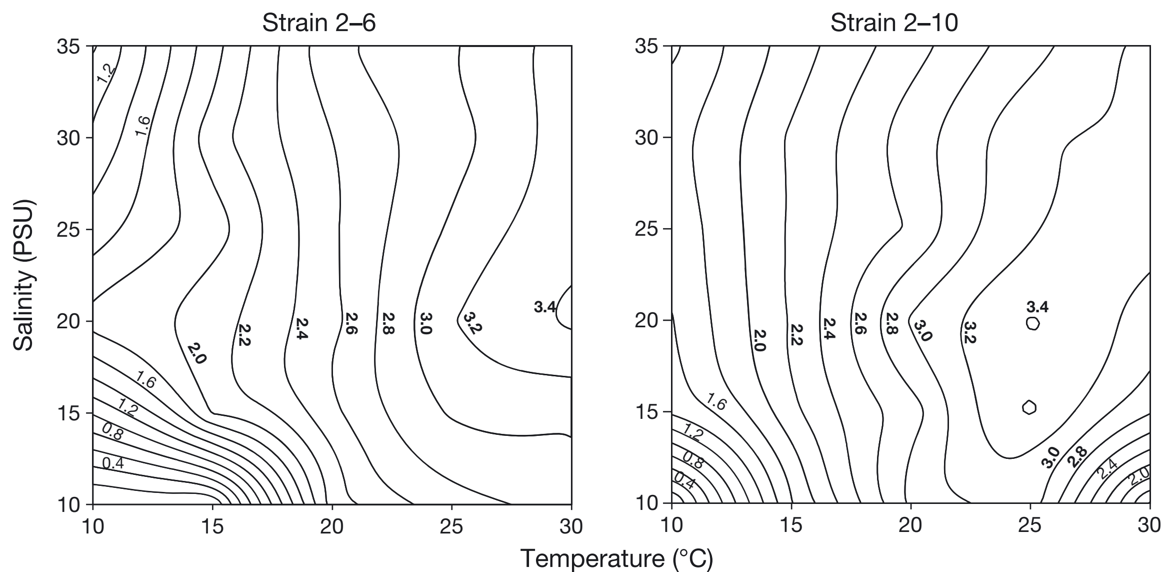

Fig. 1. Growth rates (divisions d^{-1}) of *Chaetoceros tenuissimus* cultures, strains 2-6 and 2-10, as functions of temperature and salinity

$$\text{Strain 2-6: } \mu = -5.705439 + 0.019532T^2 + -0.000392T^3 + 0.714763S + -0.021578S^2 + 0.000191S^3 + -0.014554TS + 0.000284TS^2$$

$$\text{Strain 2-10: } \mu = -7.673658 + 0.489995T + -0.000301T^3 + 0.658279S + -0.017466S^2 + 0.000209S^3 + -0.022216TS + 0.000541T^2S$$

The regression models fit the observed data with determination coefficient (R) values of 0.965 and 0.972 for the growth rates of *C. tenuissimus* strains 2-6 and 2-10, respectively. *F*-values ($F_{7,82}$) between variances and residuals obtained from the regression equations were >159 and higher than that at $\alpha = 0.001$. Akaike's information criterion values of the regression analyses were 16.7 for 2-6 strain and −33.9 for 2-10 strain, which supported goodness of fit. Therefore, these results showed the significance ($p < 0.001$) and accuracy ($R \geq 0.965$) of the regression equations for determination of *C. tenuissimus* strain growth rates at various temperatures and salinities.

Cross-reactivity tests

All of the *C. tenuissimus* strains were sensitive to CtenDNAV at all water temperatures (Fig. 2). All of the strains cultured with CtenDNAV at 30°C showed complete cell lysis at 4 dpi (Fig. 2). The periods from CtenDNAV inoculation to complete host cell lysis were shorter at higher water temperatures (Fig. 2). The complete lysis of host strain 2-6 due to CtenDNAV inoculations at 10°C required more than 10 d.

The sensitivities of the host *C. tenuissimus* strains to CtenRNAV at different water temperatures were diverse (Fig. 2). The host strains 2-10, Cten07, and Cten24 were sensitive to CtenRNAV at all water temperatures, while strain 2-6 was not lysed after the inoculations (Fig. 2). The lysis of strains 2-10 and Cten24, due to the CtenRNAV inoculations, occurred earlier at lower temperatures; however, the sensitivity of strain Cten07 showed the opposite response (Fig. 2). The complete or incomplete lysis of strains Cten01, Cten11, and Cten13 was observed at >15°C, but not at 10°C. The host strain AG07-C03 inoculated with CtenRNAV showed only incomplete lysis between 10 and 20°C (Fig. 2). Among the tested strains, we chose the host strains 2-6 and 2-10 for further experiments, because the infection specificities were different at all temperatures and lysis due to viral infections was the most obvious compared with the other strains.

Effects of water temperatures on viral proliferation

Accumulation of CtenDNAV genomes in host strain 2-6 was detected at all water temperatures, along with increasing titres (Fig. 3A,C,E & Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a073

+CtenDNAV

4 dpi

Host strains	10°C	15°C	20°C	25°C	30°C
2-10	-	-	-	+ -	+ +
2-6	-	-	-	+ -	+ +
AG07-C03	-	-	-	+ -	+ +
Cten01	-	-	-	+ -	+ +
Cten07	-	-	+ -	+ -	+ +
Cten11	-	-	+ -	+ -	+ +
Cten13	-	-	+ -	+ +	+ +
Cten24	-	-	+ -	+ +	+ +

7 dpi

Host strains	10°C	15°C	20°C	25°C	30°C
2-10	-	-	+ +	+ +	+ +
2-6	-	-	+ +	+ +	+ +
AG07-C03	-	-	+ -	+ +	+ +
Cten01	+ -	+ -	+ +	+ +	+ +
Cten07	+ -	+ +	+ +	+ +	+ +
Cten11	+ -	+ -	+ +	+ +	+ +
Cten13	+ -	+ -	+ +	+ +	+ +
Cten24	-	+ +	+ +	+ +	+ +

11 dpi

Host strains	10°C	15°C	20°C	25°C	30°C
2-10	+ +	+ +	+ +	+ +	+ +
2-6	-	+ +	+ +	+ +	+ +
AG07-C03	+ +	+ -	+ +	+ +	+ +
Cten01	+ +	+ +	+ +	+ +	+ +
Cten07	+ +	+ +	+ +	+ +	+ +
Cten11	+ +	+ +	+ +	+ +	+ +
Cten13	+ +	+ +	+ +	+ +	+ +
Cten24	+ +	+ +	+ +	+ +	+ +

Final (11-16 dpi)

Host strains	10°C	15°C	20°C	25°C	30°C
2-10	+ +	+ +	+ +	+ +	+ +
2-6	+ +	+ +	+ +	+ +	+ +
AG07-C03	+ +	+ +	+ +	+ +	+ +
Cten01	+ +	+ +	+ +	+ +	+ +
Cten07	+ +	+ +	+ +	+ +	+ +
Cten11	+ +	+ +	+ +	+ +	+ +
Cten13	+ +	+ +	+ +	+ +	+ +
Cten24	+ +	+ +	+ +	+ +	+ +

+CtenRNAV

4 dpi

Host strains	10°C	15°C	20°C	25°C	30°C
2-10	+ -	+ -	+ -	-	-
2-6	-	-	-	-	-
AG07-C03	-	-	-	-	-
Cten01	-	-	-	-	-
Cten07	-	-	-	-	-
Cten11	-	-	-	-	-
Cten13	-	-	-	-	-
Cten24	-	-	-	-	-

7 dpi

Host strains	10°C	15°C	20°C	25°C	30°C
2-10	+ +	+ +	+ -	+ -	-
2-6	-	-	-	-	-
AG07-C03	-	-	-	-	-
Cten01	-	-	-	-	-
Cten07	-	-	-	-	-
Cten11	-	-	-	-	-
Cten13	-	-	-	-	+ -
Cten24	-	+ +	+ -	+ -	-

11 dpi

Host strains	10°C	15°C	20°C	25°C	30°C
2-10	+ +	+ +	+ +	+ +	+ +
2-6	-	-	-	-	-
AG07-C03	-	-	+ -	-	-
Cten01	-	-	+ -	+ -	+ -
Cten07	-	-	+ -	-	+ -
Cten11	-	-	-	+ -	+ -
Cten13	-	-	+ -	+ -	+ +
Cten24	+ +	+ +	+ +	+ +	+ +

Final (11-16 dpi)

Host strains	10°C	15°C	20°C	25°C	30°C
2-10	+ +	+ +	+ +	+ +	+ +
2-6	-	-	-	-	-
AG07-C03	+ -	+ -	+ -	-	-
Cten01	-	+ -	+ -	+ -	+ -
Cten07	+ +	+ -	+ +	+ +	+ -
Cten11	-	+ -	+ -	+ -	+ -
Cten13	-	+ +	+ +	+ +	+ +
Cten24	+ +	+ +	+ +	+ +	+ +

Fig. 2. Susceptibility of 8 *Chaetoceros tenuissimus* strains to CtenDNAV and CtenRNAV viruses under various water temperatures at 4, 7, and 11 dpi and at the final time (11 to 16 dpi). Symbols '+ +', '+ –', and '–' indicate complete lysis, partial lysis, and not lysed, respectively

p171_supp.pdf). The signals of the strand complementary to the CtenDNAV genome in strain 2-6 cells were strongly detected at 20°C, but there were only faint signals at 15°C and 25°C (Fig. S1). The maximum yields of CtenDNAV using the host strain 2-6 at water temperatures of 15, 20, and 25°C ranged from 1.90×10^8 to 5.10×10^8 infectious units ml^{-1} (Table 1). The CtenDNAV genomes in *C. tenuissimus* strain 2-10 cells were detected at all water temperatures, which correlated with an increase of viral titres in ambient waters (Figs. 3B,D,F & S1). Accumulation of the negative-strand genome in strain 2-10 was also detected, but there was little to no accumulation at 25°C (Fig. S1). The maximum yield of CtenDNAV in host strain 2-10 under the tested temperatures was between 1.03 $\times 10^8$ and 1.90×10^8 infectious units ml^{-1} (Table 1). The CtenDNAV burst sizes ranged from 10^2 to 10^3 orders in both host strains, and burst sizes appeared rel-

Table 1. Experimental conditions and results for the viral (CtenDNAV and CtenRNAV) infection tests of *Chaetoceros tenuissimus* host strains 2-6 and 2-10 in batch culture conditions. CR_{50} = time required for cell host concentration to decrease by >50%, compared with that of the control, after viral inoculations; MOI = multiplicity of infection; dpi = days post inoculation

Virus	Temperature (°C)	Cell concentration at 0 dpi (cells ml^{-1}) Host strain 2-6	2-10	MOI Host strain 2-6	2-10	Maximum yield of viruses (infectious units ml^{-1}) Host strain 2-6	2-10	Burst size (infectious units $cell^{-1}$) Host strain 2-6	2-10	$<CR_{50}$ (dpi) Host strain 2-6	2-10
CtenDNAV	25	1.44×10^6	1.97×10^6	3.6	2.6	1.90×10^8	1.90×10^8	3.34×10^2	1.21×10^2	3	4
	20	0.98×10^6	1.39×10^6	4.9	4.9	3.50×10^8	1.03×10^8	1.43×10^3	9.60×10^2	5	8
	15	0.82×10^6	1.05×10^6	21.6	16.9	5.10×10^8	1.90×10^8	1.04×10^3	8.00×10^2	6	10
CtenRNAV	25	1.28×10^6	1.39×10^6	505	475	–	1.38×10^{10}	–	3.28×10^4	–	13
	20	1.02×10^6	1.38×10^6	3.5	3.5	–	2.55×10^{10}	–	1.86×10^4	–	10
	15	1.50×10^6	0.58×10^6	180	159	–	2.08×10^{10}	–	1.18×10^4	–	6

atively smaller at 25°C (Table 1). We determined CR_{50}, which is the time required for cell host concentration to decrease by >50%, compared with that of the control, after viral inoculations, and CR_{50} values varied depending on water temperatures and host–virus combinations. The CR_{50} values of strain 2-6 inoculated with CtenDNAV at 15, 20, and 25°C were 6, 5, and 3 dpi, respectively (Table 1); for strain 2-10, CR_{50} values at 15, 20, and 25°C were 10, 8, and 4 dpi, respectively (Table 1). Thus, the CtenDNAV CR_{50} seemed shorter at higher water temperatures.

We did not observe an increase of CtenRNAV titres, viral genome replication, or accumulation in *C. tenuissimus* strain 2-6 at any water temperature (Fig. 4A,C,E & Fig. S2 in the Supplement at www.int-res.com/articles/suppl/a073p171_supp.pdf). The accumulation of CtenRNAV genomes in *C. tenuissimus* strain 2-10 cells at 15, 20, and 25°C was detected from 1, 3, and 8 dpi, respectively (Figs. 4B,D F & S2). The signals of the CtenRNAV complementary strand in strain 2-10 cells scarcely were detected at 25°C, whereas those at lower temperatures were much stronger (Fig. S2). The maximum yield of CtenRNAV in host strain 2-10 ranged from 1.38×10^{10} to 2.55×10^{10} infectious units ml^{-1}, and the burst size was 1.18×10^4 to 3.28×10^4 infectious units $cell^{-1}$ (Table 1). The CtenRNAV CR_{50} in host strain 2-10 seemed shorter at lower temperatures; CR_{50} values were 6, 10, and 13 dpi at 15, 20, and 25°C, respectively (Table 1).

Semi-continuous culture

The fluctuations of cell concentration in the CtenDNAV-added semi-continuous culture were similar to those of the control (Fig. 5A). The initial CtenDNAV titre of 1.93×10^4 infectious units ml^{-1} decreased by 3 orders of magnitude from Day 0 to Day 4, and was then stable between 3.01 and 7.02 infectious units ml^{-1} until Day 10 when it decreased under the limit of detection (Fig. 5B). The host cell concentration in the batch culture inoculated with CtenDNAV was similar to that of the control, but it quickly declined at Day 10 (Fig. 5C). The viral titres gradually increased, and the maximum yield of CtenDNAV in the batch culture was 2.94×10^8 infectious units ml^{-1} (Fig. 5D).

During the semi-continuous culture experiment, the host cell concentration in the control culture, strain 2-10, was $2.1 \times 10^6 \pm 0.1 \times 10^6$ cells ml^{-1} (average ± standard deviation) (Fig. 6A). Meanwhile, the host cell concentration in the culture inoculated with CtenRNAV decreased gradually. The daily cell-decreasing rate from Days 2 to 11 was estimated as −7.7 ± 0.6% (linear regression analysis, slope = −0.077, $R^2 = 0.9394$, $p < 0.01$). The CtenRNAV titre at 0 dpi was 2.91×10^6 infectious units ml^{-1}; then, it rapidly increased by 2 orders of magnitude at 2 dpi and fluctuated between 1.89×10^7 and 2.26×10^8 infectious units ml^{-1} during the culture period (Fig. 6B). The host cell concentrations of the batch culture decreased rapidly at Day 10 and almost completely crashed at the end of the culture period (Fig. 6C). The CtenRNAV titre in the batch culture increased gradually, and the maximum titre was 3.47×10^{10} infectious units ml^{-1} at Day 11 (Fig. 6D).

DISCUSSION

Growth of *Chaetoceros tenuissimus*

The higher growth rates of *Chaetoceros tenuissimus* at water temperatures and salinities of 25 to 30°C and 20 to 25 PSU, respectively, are consistent with its

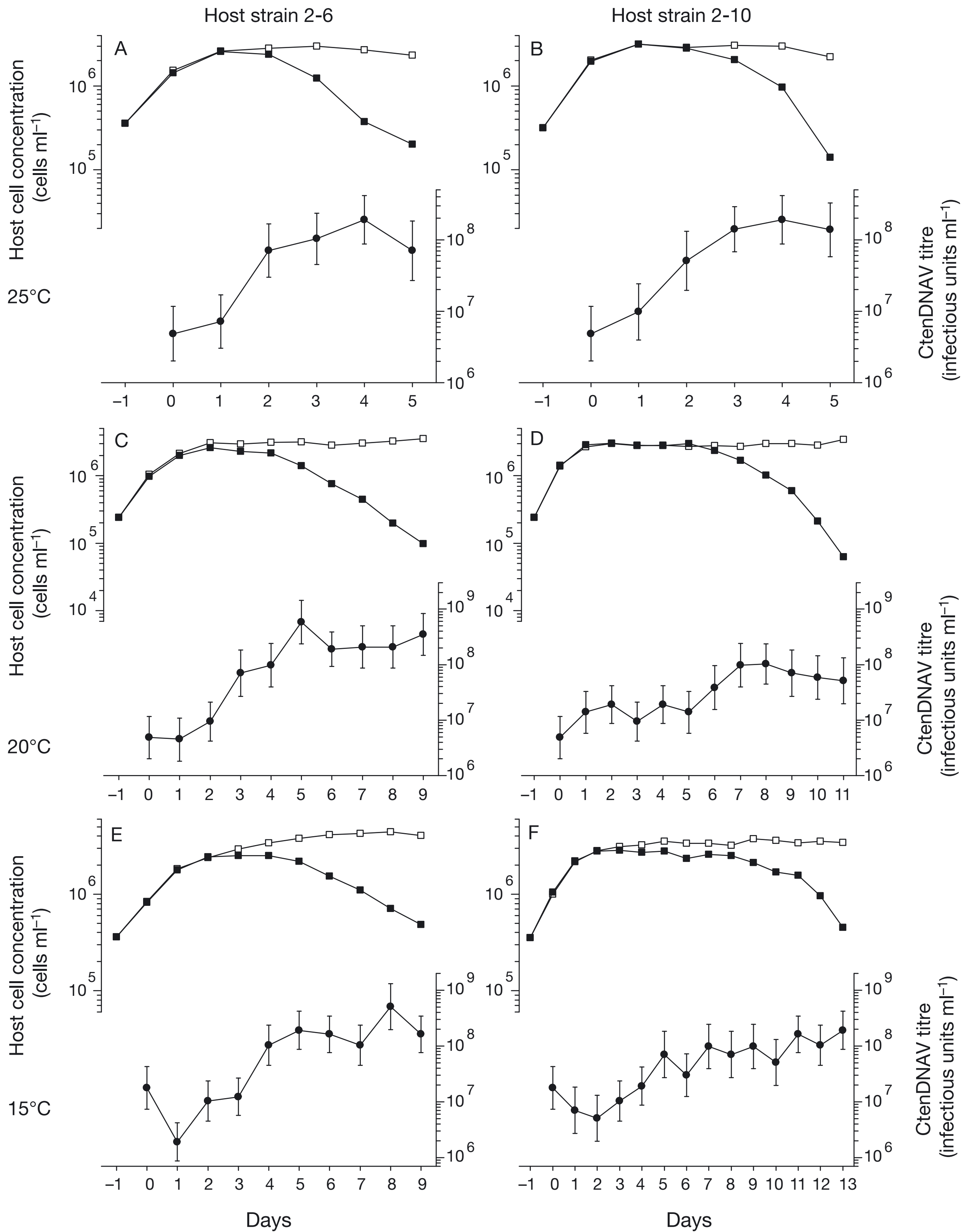

Fig. 3. Changes in cell numbers of CtenDNAV-infected (closed squares) or uninfected cultures (open squares) of *Chaetoceros tenuissimus* and viral titres (closed circles). The error bars indicate 95% confidence intervals for viral titres. (A) Host strain 2-6 at 25°C, (B) host strain 2-10 at 25°C, (C) host strain 2-6 at 20°C, (D) host strain 2-10 at 20°C, (E) host strain 2-6 at 15°C, and (F) host strain 2-10 at 15°C

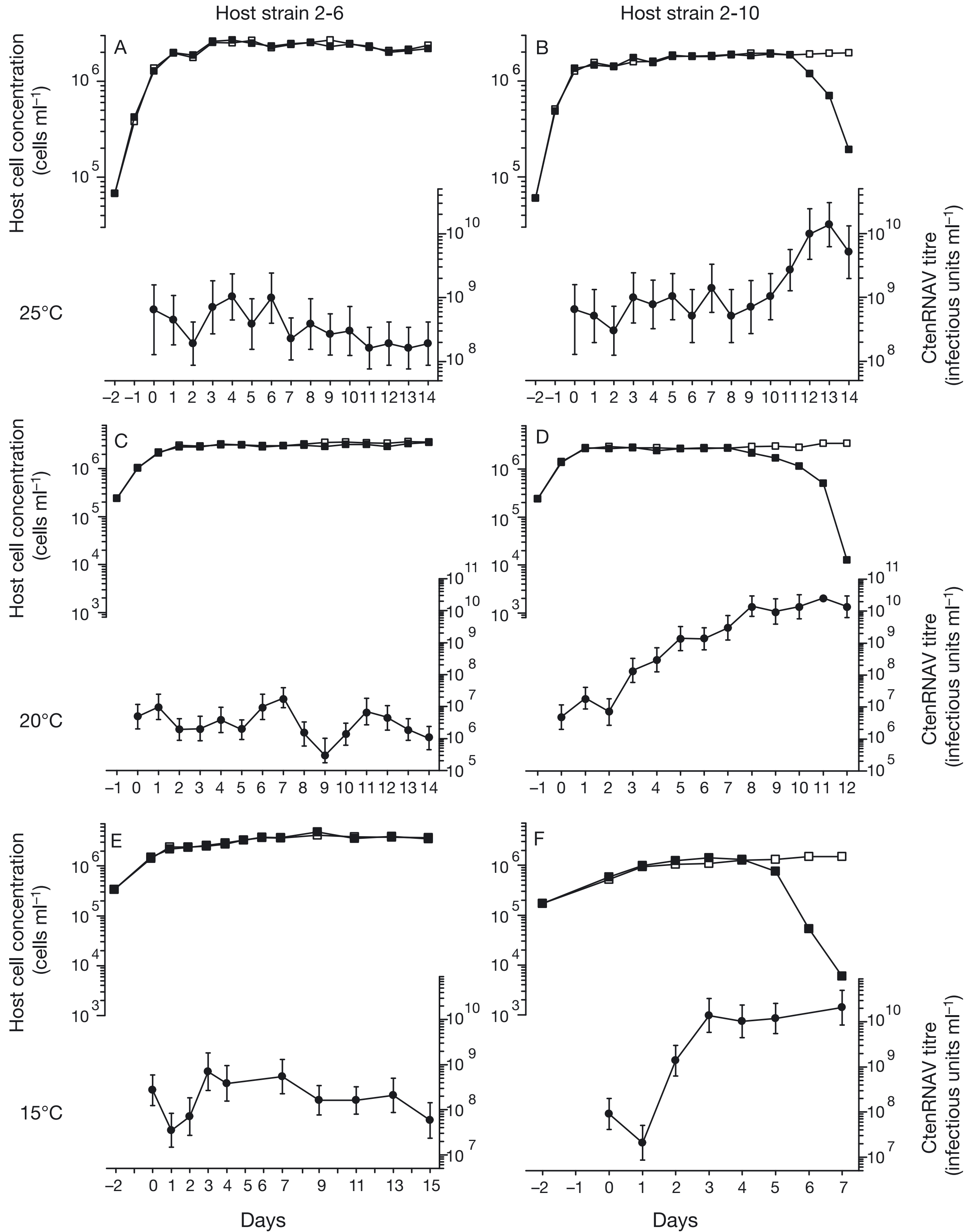

Fig. 4. Changes in cell numbers of CtenRNAV-infected (closed squares) or uninfected cultures (open squares) of *Chaetoceros tenuissimus* and viral titres (closed circles). The error bars indicate 95% confidence intervals for viral titres. (A) Host strain 2-6 at 25°C, (B) host strain 2-10 at 25°C, (C) host strain 2-6 at 20°C, (D) host strain 2-10 at 20°C, (E) host strain 2-6 at 15°C, and (F) host strain 2-10 at 15°C

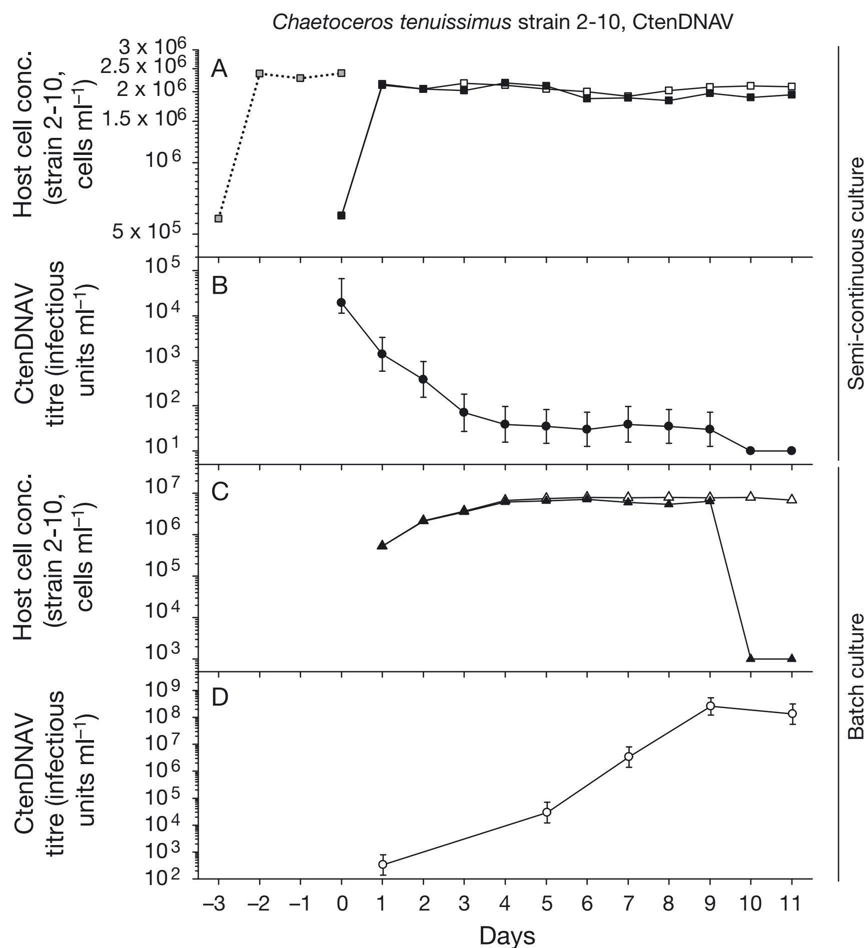

Fig. 5. Changes in cell concentrations of CtenDNAV-infected or uninfected cultures of *Chaetoceros tenuissimus* strain 2-10 and viral titres under (A,B) semi-continuous culture or (C,D) batch culture systems. Batch cultures were 75% dilutions of the remaining volume from semi-continuous cultures at day 1. (A) Temporal changes of cell concentrations in semi-continuous culture: preliminary control culture before virus inoculation (···■···), uninfected culture (□), and CtenDNAV-inoculated culture (■). (B) CtenDNAV titres in the inoculated semi-continuous culture (●). (C) Uninfected culture (△) and CtenDNAV-inoculated culture (▲). (D) CtenDNAV titres in the inoculated batch culture (○). The error bars indicate 95% confidence intervals for viral titres

known dynamics in Hiroshima Bay, where the organism grows well and forms blooms in the summer season (≥25°C) during July to September (Tomaru et al. 2011a). Further, salinities in Hiroshima Bay often decrease to <25 PSU because of rainfall and the resultant discharge of riverine waters during summer seasons (Kamiyama & Tsujino 1996). Moreover, *C. tenuissimus* growth was detectable at lower temperatures except in combination with low salinities (≤15 PSU). This result is reasonable considering that the organism is observable throughout the year, including in low-temperature seasons, in Hiroshima Bay (8 to 15°C and approximately 30 PSU; Y. Tomaru unpubl. data).

CtenDNAV ecology in nature

The period for a host culture crash after CtenDNAV infection was shorter at higher water temperatures (Table 1 & Fig. 3), which are suitable for host growth. However, in this study, the MOI varied among the experiments and there were no replicates, making it difficult to compare the CR_{50} values among the experiments. According to previous reports for CtenRNAV, the CR_{50} at 15°C in host strain 2-10 under a late logarithmic growth phase appeared to be 8 dpi where the MOI was 12.1 (Shirai et al. 2008); thus, a comparison with our results indicates that a 10-fold difference of MOI might affect 30% error for the CR_{50}. A higher initial MOI would be expected to result in an earlier decline in host cell concentration, e.g. *Phaeocystis pouchetii* and its large dsDNA virus with an MOI of 0.15 to 50 (Bratbak et al. 1998). Van Etten et al. (1983) also suggested that higher MOI affects cell lysis without viral infections. Consequently, the present data must be cautiously evaluated, and the absolute value of CR_{50} could change because of MOI differences. The CtenDNAV CR_{50} was shorter at higher water temperatures, even though the MOI was higher at lower temperatures (Table 1). Under these conditions, based on the combined results of the cross-reactivity tests (Fig. 2), the relationship between CtenDNAV and water temperature might not be affected by the difference in MOI. Thus, *C. tenuissimus* cells under suitable growth conditions at higher water temperatures would be vulnerable to CtenDNAV infections.

At any water temperature, the latent periods of CtenDNAV were 2 or 3 d, based on the increased titres (Fig. 3). The CtenDNAV burst sizes were not large at higher temperatures (Table 1). Therefore, the rapid decrease of host cell concentration after CtenDNAV inoculation at higher water temperatures cannot be explained by changes in the latent period and the burst size. Considering that the CtenDNAV CR_{50} varied with respect to water temperature, the percentage of cells that are permissive to viral infection would be larger at higher water temperatures. In

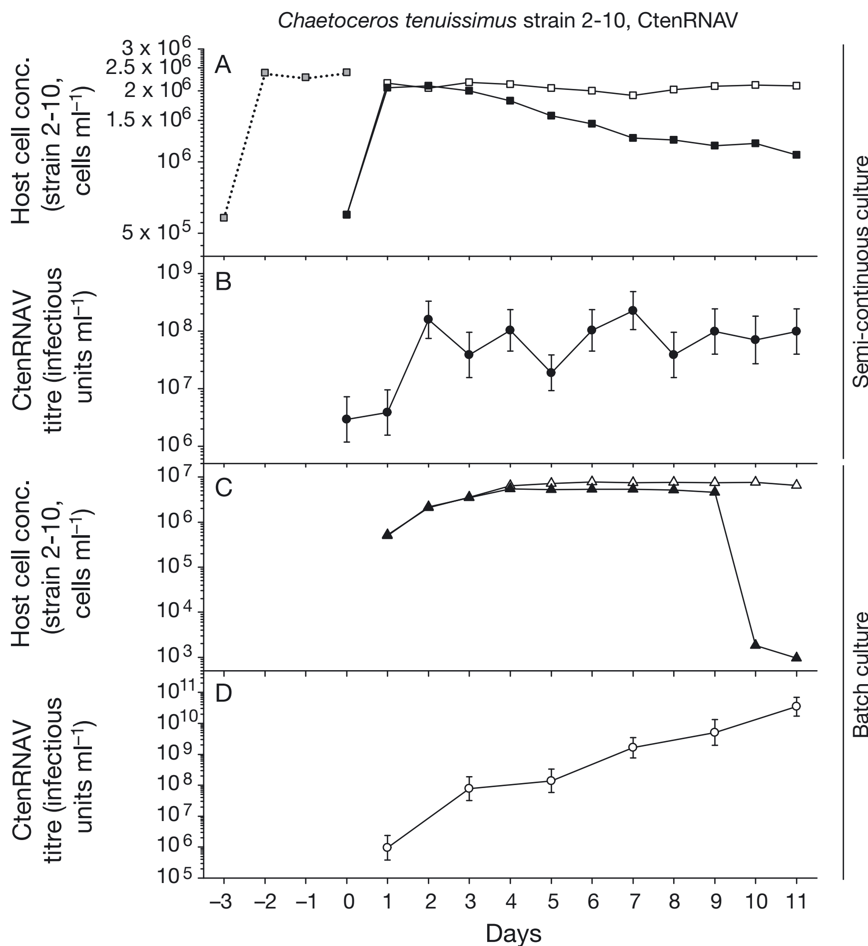

Fig. 6. Changes in cell concentrations of CtenRNAV-infected or uninfected cultures of *Chaetoceros tenuissimus* strain 2-10 and viral titres under (A,B) semi-continuous culture or (C,D) batch culture systems. Batch cultures were 75% dilutions of the remaining volume from the semi-continuous cultures at day 1. (A) Temporal changes of cell concentrations in semi-continuous culture: preliminary control culture before virus inoculation (···■···), uninfected culture (□), and CtenRNAV-inoculated culture (■). (B) CtenRNAV titres in the inoculated semi-continuous culture (●). (C) Uninfected culture (△) and CtenRNAV-inoculated culture (▲). (D) CtenRNAV titres in the inoculated batch culture (○). The error bars indicate 95% confidence intervals for viral titres

natural environments, CtenDNAV proliferation would be associated with the host cell growth, dependent on water temperature; thus, the virus would be dominant in the summer season.

Host cell decline due to CtenDNAV inoculations was not observed in the semi-continuous culture experiment, yet the virus concentrations were stable for at least 7 d at a low level, approximately 10^1 infectious units ml^{-1}. This result indicates that CtenDNAV infections and proliferation occur in host strain 2-10, but infections occur at an extremely low level. The CtenDNAV burst size, when infecting host strain 2-10, was estimated to be approximately 10^2 infectious units $cell^{-1}$ (Table 1), and the latent period (i.e. significant increases of viral titre) at 15°C was at least 3 d (Fig. 3F). Considering these results, the theoretical percentage of CtenDNAV-sensitive cells in the logarithmic growing host population at 15°C would be <0.001%. However, the MOI in this experiment was 2 orders lower than that of CtenRNAV, and this difference could affect the interpretation of the percentage of CtenDNAV-infection-permissive cells. The titre of CtenDNAV in the semi-continuous culture might continuously decrease but remain even after Day 10 at the level under the limit of the detection, <3.01 infectious units ml^{-1}. Although CtenDNAV would not be dominant during seasons of lower water temperature, our results indicate that the virus could survive as long as the host populations exist in the water column. Furthermore, virus infectivity is retained under lower temperatures (Tomaru et al. 2011b), which contributes to virus survival during winter seasons.

CtenRNAV ecology in nature

Although direct comparison of the CR_{50} values is difficult because of the MOI differences, the CtenRNAV infections in host strain 2-10 appeared to be effective at lower temperatures, at which host growth was not maximal (Table 1, Figs. 1, 2 & 4). According to many microalgal virus studies, viral proliferations are enhanced where the host growth conditions are optimal (Van Etten et al. 1983, Bratbak et al. 1998, Nagasaki & Yamaguchi 1998). Notably, suitable environmental conditions for host growth did not necessarily correlate with CtenRNAV proliferation. This result differs from that of CtenDNAV and might explain how the 2 viruses share the host populations with little competition and coexist in the same environment.

The lytic intensities and periods of *C. tenuissimus* strain population decline due to CtenRNAV inoculation were highly diverse across water temperatures (Fig. 2). Viruses use the host's metabolic system and

materials for their proliferation. Therefore, sensitivity to variations of the host strains and to water temperature might be related to host strain-specific physiological characteristics; yet, this assumption warrants further research. The heterogeneity of host strain susceptibility to CtenRNAV, with respect to water temperature, would allow CtenRNAV to survive all seasons in nature. The temperature effects on the host–CtenRNAV system appear to be highly complex, more so than those in the case of the DNA virus; consequently, this system requires further research.

Effect of host growth phase on viral infection

The semi-continuous culture showed that only 7.7% of cells were CtenRNAV infection-permissive in the logarithmic growth populations, even though the host strains potentially permit virus infection. This result indicates that the cell conditions in the logarithmic growth phase are diverse with regard to viral infectivity. The percentage of cells that are virus infection-permissive might increase along with stationary phase progression. In batch culture experiments in the present study, the viral proliferation signals and/or increase of the titre did not directly correlate with decrease of the host cell densities during logarithmic growth phases (Figs. 3, 4, S1 & S2), and this result can be explained, because there was little virus-induced cell death compared with vigorous host cell growth. Although the host defence mechanisms are still unknown, the success of CtenRNAV infection and proliferation in *C. tenuissimus* cells is likely related to host cell physiological conditions, especially growth phases. In natural environments, *C. tenuissimus* blooms often sustain population sizes even in the presence of infectious viruses (Tomaru et al. 2011a), which can also be partially supported by the above reason.

Implications

The coexistence of viruses that share the same host species has been observed in many aquatic environments (Waterbury & Valois 1993, Brussaard et al. 1996, Sahlsten 1998, Tomaru et al. 2004, 2009, Baudoux & Brussaard 2005). The mechanisms to facilitate virus coexistence or niche partitioning can be explained by a complex relationship between diverse virus specificity for the host strain and viral susceptibilities of the host strains (Flores et al. 2011), which may reduce competition among various virus species; consequently, the difference in CtenDNAV and CtenRNAV infectivities substantiate that explanation. Furthermore, the results of the present study suggest that these diatom viruses occupy separate niches because of water temperature-dependent survival strategies. The niche partitioning among viruses in natural environments might occur because of not only their differential host ranges, but also their environmental conditions.

High viral concentrations in natural environments are expected when the host cells are abundant, which occurs under appropriate environmental conditions (Suttle & Chan 1994, Tarutani et al. 2000, Tomaru et al. 2007). In general, viruses proliferate by co-opting their host's physiological functions; therefore, environmental factors that affect host metabolism are indirectly related to viral proliferation. The results of CtenDNAV experiments agree with this theory; however, CtenRNAV proliferation did not necessarily concur with this theory. These results indicate an inconsistency in the dominant virus and host organism abundance in nature.

Finally, the growth rates of the diatom host strains were significantly affected by salinity level, warranting a detailed future evaluation of the salinity effects on the diatom host–virus relationship. In addition to water temperature and salinity, other factors should be studied. In several marine phytoplankton–virus systems, such as *Phaeocystis globosa* and *Micromonas pusilla* (Prasinophyceae) and their infectious viruses, light is an important factor determining their interactions, i.e. infectivity, latent period, and burst size (Baudoux & Brussaard 2008). Furthermore, nutrient limitations are significant for phytoplankton growth and simultaneously affect viral proliferation (Bratbak et al. 1998). Therefore, we plan to evaluate the relationship between the effects of more environmental factors on host growth and viral proliferation for further understanding of the role of diatom viruses on host dynamics.

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