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Mortality and herpesvirus infections of the Pacific oyster *Crassostrea gigas* in Tomales Bay, California, USA

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ABSTRACT: Seed losses of Pacific oysters *Crassostrea gigas* have been associated with an ostreid herpesvirus-1 (OsHV-1) in Europe, and in 2002, a similar OsHV was detected in Tomales Bay, California, USA. In May of 2003, 5 stocks of seed Pacific oysters were planted at 2 sites (Inner Bay and Outer Bay) in Tomales Bay and monitored for mortality, presence/prevalence of OsHV (using polymerase chain reaction [PCR] and histology), and growth. Temperature (°C) and salinity data were collected every half an hour at each site. OsHV was detected at both the Inner and Outer Bay sites on the same sample date and mean temperature predicted OsHV presence ($p < 0.005$). High levels of mortality occurred 2 wk (Inner Bay site) and 4 wk (Outer Bay site) after OsHV detection. OsHV presence predicted mortality ($p = 0.01$). Temperature maximums and overall temperature exposure were greater at the Inner Bay site and may explain why mortality affected these oysters sooner than oysters planted at the Outer Bay site. Differences in cumulative mortality were significant among stocks ($p < 0.0001$), but not between sites ($p > 0.05$). OsHV prevalence was similar among stocks ($p > 0.05$) and between sites ($p > 0.05$). No evidence of herpesvirus-induced Cowdry type A nuclear inclusions or other pathogens were observed. Changes in tissue and cellular architecture including dilation of the digestive tubules and nuclear chromatin margination and pycnosis were observed in OsHV-infected oysters, consistent with previously observed OsHV infections. Stocks with smaller oysters had higher mortality rates than those with larger oysters; growth rate did not correlate with mortalities ($p > 0.05$). Taken together, these data suggest that the OsHV may cause or act in synergy with temperature to kill Pacific oyster seed in Tomales Bay, but further investigation of OsHV etiology in seed oysters is needed.

KEY WORDS: Pacific oyster · Oyster herpesvirus · Mortality · Tomales Bay · California · Temperature

INTRODUCTION

Losses of Pacific oyster *Crassostrea gigas* seed grown in Tomales Bay, California, USA, were first observed in 1993 when up to 90% losses of seed oysters and high losses of production oysters occurred (Friedman et al. 1997, Cherr & Friedman 1998). Prior to 1993, typical losses over an 18 mo culture cycle averaged 8 to 35% (Glude 1974, J. Finger pers. comm.). In contrast, over the past 10 yr short pulses of mortality have occurred during the summer months (typically May to November), reaching up to 100% over a 2 wk period, and particularly affecting newly planted seed <2 cm in size (Cherr & Friedman 1998, C. S. Friedman unpubl. data). Biotic (e.g. phytoplankton abundance) and abiotic factors (e.g. extreme temperature fluctuations) have been observed in association with oyster losses in California (Cherr & Friedman 1998, C. S. Friedman unpubl. data).

Larval Pacific oyster losses were noted in 1991 in association with an ostreid herpesvirus (OsHV-1) in France (Nicolas et al. 1992) and a morphologically similar OsHV in New Zealand (Hine et al. 1992). OsHV-induced mortalities in larvae are short in dura-

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tion (between 4 d and 2 wk), cause high levels of losses (up to 100%), and are associated with temperatures in excess of 25°C (for example: Le Deuff et al. 1996 or Arzul et al. 2001b). Interestingly, OsHV infects multiple hosts, which is unique among members of the family *Herpesviridae* (Davison 2002). Larval Pacific oysters *Crassostrea gigas* (Hine et al. 1992, Nicolas et al. 1992), Manila clams *Venerupis* (=*Ruditapes*) *philippinarum* (Renault & Arzul 2001, Renault et al. 2001), European clams *Ruditapes decussates* (Arzul et al. 2001c, Renault & Arzul 2001), flat oysters *Tiostrea chilensis* (Hine et al. 1998), European oysters *C. angulata* (Arzul et al. 2001b), and French scallops *Pecten maximus* (Arzul et al. 2001a) are all susceptible to OsHV infection-induced mortalities. Mortality of juvenile Pacific oysters and European flat oysters *Ostrea edulis* has been associated with OsHV (Comps & Cochennec 1993, Renault et al. 1994, 2000a, Friedman et al. 2005), but the ability of the virus to kill juvenile oysters is not well documented. OsHV infections in seed are associated with catastrophic losses of smaller seed oysters over a short duration during warm water periods in France (Renault et al. 2000a), but unlike losses in France, only Pacific oysters are dying in association with OsHV in Tomales Bay. Adult bivalves *C. gigas* (Arzul et al. 2002), *O. angasi* (Hine & Thorne 1997), and the French scallop *P. maximus* (Arzul et al. 2001a) are also susceptible to infection, but mortality has not been documented in adult hosts.

Due to the timing and similarities of the mortalities in France and New Zealand to those occurring in Tomales Bay, a polymerase chain reaction (PCR) (Renault et al. 2000b, Renault & Arzul 2001) and an *in situ* hybridization (ISH) (Lipart & Renault 2002) were used to test for the presence of OsHV DNA in tissues (Friedman et al. 2005) and archived histological sections (C. S. Friedman unpubl. data.), respectively. In 2002, following oyster mortality events, OsHV infections were detected in Tomales Bay by PCR amplification of genomic OsHV DNA and subsequent sequence analysis (Friedman et al. 2005). In addition, OsHV-infected connective tissue cells were observed using a specific ISH probe for OsHV preceding and during mortality events in 1995 and after 2002 mortality events (C. S. Friedman unpubl. data).

OsHV-induced mortality of Pacific oyster larvae in France and New Zealand is well documented. However, the association between seed mortalities and OsHV infection in France (Renault et al. 2000a) and in Tomales Bay, California (Friedman et al. 2005), still warrant further examination. The relationship between the presence of this virus and mortality events in Tomales Bay is currently unknown. In this study, we combined a field experiment with laboratory analyses to determine whether a relationship exists between (1) the presence of OsHV and timing of mortality events in Tomales Bay, and/or extreme temperature such as minimums, means, or maximums, (2) OsHV prevalence and differential mortality, and (3) oyster growth and differential mortality.

MATERIALS AND METHODS

Field study. Five stocks of Pacific oyster seed were obtained from 3 different suppliers: (1) Strait of Georgia, Washington State (WA-1); (2) Hood Canal, Washington State (WA-2, a diploid oyster stock, and WA-3, a triploid oyster stock produced in the same hatchery); and (3) Yaquina Bay, Oregon (pedigreed oyster lines: OR-1, predicted to be a high performing line based on previous survival and growth, and OR-2, predicted to be a poor performing line, C. Langdon, Oregon State University, pers. comm.) donated by the Molluscan Broodstock Program.

Oyster height (length) and weight were measured prior to planting oysters. Differential height ($p < 0.0001$) and weight ($p < 0.0001$) were observed among stocks (Table 1).

Triplicate bags of 1000 oysters from each source were planted at the Outer (May 16) and Inner (May 17) Tomales Bay sites (Fig. 1) at the +1.0 ft (0.3 m) level in ¼ inch (6 mm) mesh individual Nytex bags attached to metal racks 0.3 m above the substrate. Each replicate bag was individually tagged and handled separately. Mortality was quantified bimonthly by enumerating the number of dead oysters out of a random sample of 100 oysters per bag. Dead oysters were identified by the presence of open and gaping valves with or without some amount of tissue remaining. Bags with new mortalities often had either gaping oysters with remaining tissues in the shell or shells that were completely clean. Dirty shells or those filled with mud were considered older mortalities. Subsequently, all dead oyster shells were removed and 25 oysters were

Table 1. *Crassostrea gigas.* Size in height and weight (±1 SD) for 5 stocks planted in Tomales Bay, California, in May 2003

Measurement	Oyster stock				
	WA-1	WA-2	WA-3	OR-1	OR-2
Height (cm)	2.00 ± 0.27	1.26 ± 0.22	1.22 ± 0.19	0.91 ± 0.34	0.99 ± 0.26
Height range	1.49 – 2.91	0.78 – 1.95	0.89 – 1.84	0.16 – 1.99	0.51 – 1.64
Weight (g)	1.22 ± 0.40	0.30 ± 0.78	0.30 ± 0.13	0.16 ± 0.17	0.11 ± 0.078
Weight range	1.50 – 2.38	0.10 – 0.71	0.11 – 0.90	0.03 – 0.75	0.03 – 0.44

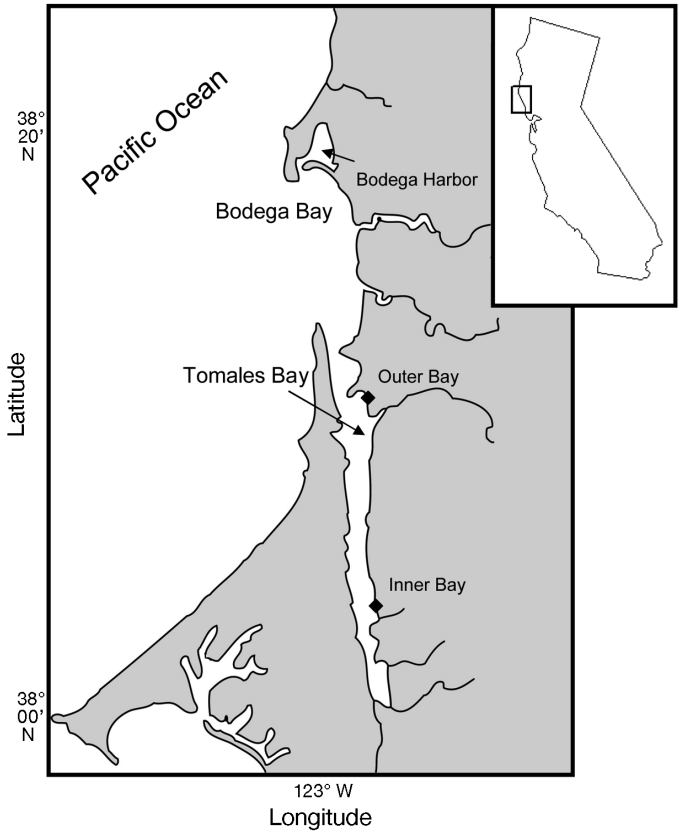

Fig. 1. Map of California, USA, with an enlargement of Tomales Bay and surrounding areas. Site locations in Inner and Outer Bay are labeled with black diamonds

collected from each bag (or 75 from each stock) and transported to the Bodega Marine Laboratory (BML) for morphological measurements and the analyses described below. Continuous water quality data loggers recorded environmental data, including water temperature (°C) and salinity every 30 min at each site (YSI).

Laboratory methods. After returning to BML, oyster height (cm) and weight (g) were measured for each of the 25 oysters sampled per bag. Three of the stocks (WA-1, WA-2, and WA-3) were selected for histological and PCR analyses.

Histology: Thirty oysters from each of the 3 stocks (10 per bag) were removed from their shells and two 3 mm cross sections were excised ventral to the labial palps. Each cross section included gill, mantle, and digestive gland. One cross section was placed in 95% ethanol for PCR analysis as described below. The second section was placed in Invertebrate Davidson's solution (Shaw & Battle 1957) for 24 h and processed for routine paraffin histology (Luna 1968). De-paraffinized 5 µm tissue sections from a subset of oysters were stained with hematoxylin and eosin and viewed by bright field microscopy using a Nikon E600 microscope. Stained tissue sections were examined for morphological changes suggestive of herpes virus infection, other infectious agents, and anomalies.

Nucleic acid extraction: Initially, nucleic acid was extracted from 2 pools of 5 oysters per bag or a total of 6 pools per group (n =18 pools per site); equal amounts of tissue from each oyster were included in each pool. When OsHV presence was established on the July 29/30 sampling dates, individual oysters were extracted for analysis on the next sample date, August 12/13 (n =10 oysters per bag, 30 per group, or a total of 90 per site). For both pooled and individual samples, approximately 25 mg of tissue was placed into a Qiamp Mini DNA Kit (Qiagen) and processed according to the manufacturers' protocol.

PCR: *Oyster herpesvirus:* The nested PCR for the OsHV employed the primers and PCR conditions of Renault et al. (2000b) as modified by Friedman et al. (2005). Positive control OsHV DNA was obtained from OsHV of infected Tomales Bay oysters (Friedman et al. 2005). Briefly, each 25 µl reaction contained 10× PCR buffer, 0.2 mM dNTP mix, 2.5 mM $MgCl_2$, 8 µg BSA, 4 pmol of each primer and 1.0 U *Taq* Polymerase (Promega), and genomic oyster DNA (either 50 or 500 ng). Duplicate reactions of each sample were run at 2 concentrations (50 and 500 ng) to maximize OsHV detection (Friedman et al. 2005). Primers A3 (5′GCCAACCGTTGGAACCATAACAAGCG 3′) and A4 (5′GGGAATGAGGTGAACGAAACTATAGACC 3′) were used to amplify a 1001 bp segment of the viral genome using an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final elongation step at 72°C for 5 min. One µl from the A3/A4 reaction was used as template in the nested reaction in which primers A5 (5′CGCCCCAACCACGATTTTTCACTGACCC 3′) and A6 (5′CCCGCAGAATAGGATGAGATTTG 3′) were used to amplify a 917 bp segment of the OsHV genome using the same thermal profile listed above. Products were separated on 1.0% agarose gels containing 0.1 mg ml^{-1} ethidium bromide and visualized using a UV transilluminator (Renault et al. 2000b).

Universal small subunit (*SSU*) *genes:* The presence of amplifiable nucleic acid was confirmed in all samples that tested negative for OsHV by PCR using universal SSU ribosomal RNA gene primers (Le Roux et al. 1999; their Fig. 1B). Primers CS1 (5′GTACGGGGAATCAGGGTTCG 3′) and CAS1 (5′GGTGCCCTTCCGTCAATTCC 3′) were employed using the modified conditions of Friedman et al. (2005). Each 20 µl reaction contained 10 × PCR buffer (10 mM Tris, pH 8.3; 50 mM KCl), 2.5 mM $MgCl_2$, 8 µg BSA, 0.2 mM dNTP mix, 10 pmol of each primer, 1 U *Taq* Polymerase (Pro-

mega) and 1 µl of template DNA. Nucleic acid amplification were performed with an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final elongation at 72°C for 10 min. PCR products were separated as mentioned above with an expected product size of 810 bp.

Data analysis. Both instantaneous and cumulative mortality were calculated. Instantaneous mortality rates were calculated as the number of oysters that died over the sample period divided by the number of oysters alive at the beginning of the period. Total inferred mortalities were divided by the number of oysters originally outplanted. Mortality was arcsine-transformed for all analyses to give weight to percentage data (Zar 1999). Differences in mortality among oyster stocks and sites were assessed using an ANOVA, and identified using a Tukey test (Zar 1999).

Environmental data, including salinity and temperature data, were compared at the Outer and Inner Bay sites. Mean daily salinity and temperature were found to be serially auto-correlated with the same first order auto-correlation. Therefore, salinity and temperature data were compared using a *z*-test correcting for serial correlation (Ramsey & Schafer 2002, p. 444–445) corrected with a Bonferroni p-value. For each site, temperature minimum, mean, and maximum were computed every 2 wk between sampling periods. Hours exposed to temperatures over 16, 18, 20, 22, and 24°C were quantified monthly at each site and compared using a paired *t*-test.

In order to further assess the influence of temperature on oyster survival, the total number of hours over 16 to 25°C (in 1°C increments), and the degree hours per day that oysters were exposed to in 2003 was calculated. The Spearman Rank Correlation tested if a linear relationship existed between total hours at temperatures from 16 to 25°C or the number of hours per day at each temperature and mortality at each site. In addition, temperature and mortality data collected during 2001, 2002 (C. S. Friedman unpubl. data), and 2003 at the Inner and Outer Bay sites were compared at each site.

OsHV prevalence, mortality, and environmental parameters were compared by using a variety of analyses. Logistic regression was used to identify which parameter or parameters, including temperature (minimum, mean, and maximum), salinity, and outplant site were associated with OsHV presence (S+ 7.0; Insightful Corporation). Logistic regression was also used to identify which of the following parameters: temperature (minimum, mean, and maximum), salinity, outplant site, and OsHV presence were associated with mortality. Individual sites were also tested using both of these models. Chi-square tests were used to test for the statistical significance of a given predictor variable, as long as the model was not overdispersed. In the case of overdispersion, an *F*-test (which uses a scaling factor that accounts for the overdispersion) was used. OsHV prevalence was compared among oyster stocks and between sites and relative to cumulative mortality. OsHV prevalence was arcsine transformed and compared between oyster stocks and sites using an ANOVA; differences were identified by using a Tukey Test (Zar 1999). Prevalence and cumulative mortality data from individual bags were tested using a linear model with log-transformed data (Neter et al. 1996).

Differences in final shell height and weight were examined among stocks and sites by ANOVA; differences were identified by using a Tukey test (Zar 1999). Additionally, growth rate was calculated for both height and length data. Growth rate of oyster height was inferred by the slope of the growth curve over the duration of the experiment. Standardized growth rates (average weight gain d^{-1}) were calculated over the experiment period (May 16/17 to September 9/10) and between sample periods using the following formula from Rowland et al. 2004:

$$\ln(Mx/Mo)/X \times 100 = \%\ \text{growth}\ d^{-1}$$

where Mx = average oyster weight at the end of the period, Mo = average oyster weight at the beginning of the period, X = the number of days in the sample period.

Pearson's Moment Correlation tested whether a linear relationship existed between overall growth rate (based on length and the standardized growth rate) and cumulative mortality (SPSS 12.0; SPSS).

RESULTS

Oyster mortality

Oyster mortality was minimal at the Inner Bay site until late July and at the Outer Bay site until late August (Table 2). Fresh mortalities were observed in all stocks at the Inner Bay site on July 29 (range of instantaneous mortality: 3.30 to 7.00%), and instantaneous mortality between July 30 and August 12 was high (5.70 to 64.00%). Mortality continued to affect all stocks to varying degrees until the end of the experiment on 9/9/2003. At the Outer Bay site, mortality was absent until August 13, and initially mortality occurred only in one group (OR-1, 7.01 ± 12.14% [mean ± 1SD]). Between August 13 and 27 mortality was moderate to high in all stocks (10.04 to 70.00% losses) except for the WA-3 group, where mortality was absent. All groups were affected between August 28 and September 10 (4.67 to 39.00% losses).

Table 2. *Crassostrea gigas.* Instantaneous and cumulative mortalities of Pacific oysters (5 stocks) in Tomales Bay in 2003. Instantaneous mortality: proportion of oysters (±1 SD) that died since the last sample date; cumulative mortality: number of oysters (±1 SD) which died through the whole experiment divided by the original number in each bag (1000)

Site	Date (mo/d/yr)	Percent mortality				
		WA-1	WA-2	WA-3	OR-1	OR-2
Inner Bay	Instantaneous					
	06/03/2003	0	0	0	0	0
	06/16/2003	0	0	0	0	0
	07/01/2003	0	0	0	0	0
	07/15/2003	0	0	0	4.00 ± 6.93	0.60 ± 0.56
	07/29/2003	7.00 ± 4.58	3.30 ± 3.51	5.70 ± 4.04	6.00 ± 2.29	6.00 ± 4.09
	08/12/2003	21.30 ± 11.02	39.00 ± 12.28	5.70 ± 2.46	64.00 ± 18.16	31.60 ± 3.50
	08/28/2003	3.00 ± 5.17	14.40 ± 4.72	0	17.30 ± 22.62	18.30 ± 7.03
	09/09/2003	1.30 ± 1.53	3.00 ± 2.65	1.00 ± 1.00	16.70 ± 16.17	5.00 ± 4.36
	Cumulative	26.17 ± 15.88	44.02 ± 10.08	10.52 ± 2.63	64.92 ± 15.88	43.25 ± 6.72
Outer Bay	Instantaneous					
	06/04/2003	0	0	0	0	0
	06/13/2003	0	0	0	0	0
	06/30/2003	0	0	0	0	0
	07/14/2003	0	0	0	0	0
	07/30/2003	0	0	0	0	0
	08/13/2003	0	0	0	7.01 ± 12.14	0
	08/27/2003	10.04 ± 7.83	13.04 ± 7.18	0	42.67 ± 26.13	70.00 ± 2.00
	09/10/2003	22.67 ± 3.79	20.33 ± 9.71	4.67 ± 1.53	39.00 ± 23.64	30.00 ± 18.19
	Cumulative	25.13 ± 7.80	25.73 ± 3.03	3.03 ± 1.72	58.08 ± 9.93	66.58 ± 3.40

Cumulative mortality ranged from 10.52 to 64.92% at the Inner Bay site and 3.03 to 66.58% at the Outer Bay site. Differential cumulative mortality occurred among stocks (Fig. 2; $p < 0.0001$) but not between sites ($p > 0.05$). Oyster stocks grown at each site had similar mortality, and a stock by site by mortality interaction was not observed ($p > 0.05$).

Environmental exposures and mortality

Environmental exposures, measured by temperature and salinity, were different for oysters grown at Inner and Outer Tomales Bay in 2003. Salinity was greater (32.75 ± 1.66 [mean ± 1SD]) at the Outer Bay site ($p < 0.0001$) than at the Inner Bay site (29.96 ± 2.40 ppt). Oysters grown at the Inner Bay site were exposed to higher maximum temperatures (Fig. 3), and longer hours of elevated water temperatures at 18°C ($p < 0.01$), 20°C ($p < 0.05$), and 22°C ($p < 0.02$) (but not 16°C, $p > 0.05$ or 24°C, $p > 0.05$) than those grown at the Outer Bay site (Fig. 4). Mean temperature exposure was the same at the Inner and Outer Bay sites ($p > 0.05$). On July 15, a temperature spike of 27.13°C occurred at the Inner Bay site followed by a 3 wk period of elevated water temperatures and daily temperature maxima approaching 24°C (Fig. 5). Mortality first occurred at the Inner Bay site approximately 2 wk after this temperature spike (July 30), and mortality was correlated with temperatures between 16 and 25°C ($p < 0.05$ for each temperature, Table 3). Mortality occurred at the Outer Bay site after a temperature maximum of 22.98°C (August 4) and correlated with elevated temperatures (16 to 22°C) ($p < 0.05$ for each temperature, Table 3).

Mortality and different temperature exposures were correlated at Inner Bay and Outer Bay sites in 2003. When 3 yr of temperature and mortality were examined (2001–2003), mortality and temperature exposure at the Inner Bay site correlated only with total hours of temperature in excess of 24°C (correlation coefficient $C = 0.658$, $p < 0.05$) and 25°C ($C = 0.700$, $p < 0.05$) and degree hours per day in excess of 24°C ($C = 0.794$, $p < 0.01$) and 25°C ($C = 0.697$, $p < 0.05$). Mortality at the Outer Bay site in the years 2001–2003 was correlated with temperatures in excess of 16°C ($C = 0.711$, $p < 0.05$).

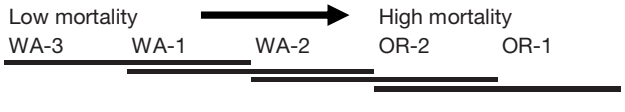

Fig. 2. *Crassostrea gigas.* Statistical differences ($p < 0.0001$) in cumulative mortality among the 5 stocks outplanted in Inner and Outer Tomales Bay, in 2003. Included is data from both Inner and Outer Bay sites as no differences were observed between sites ($p > 0.05$)

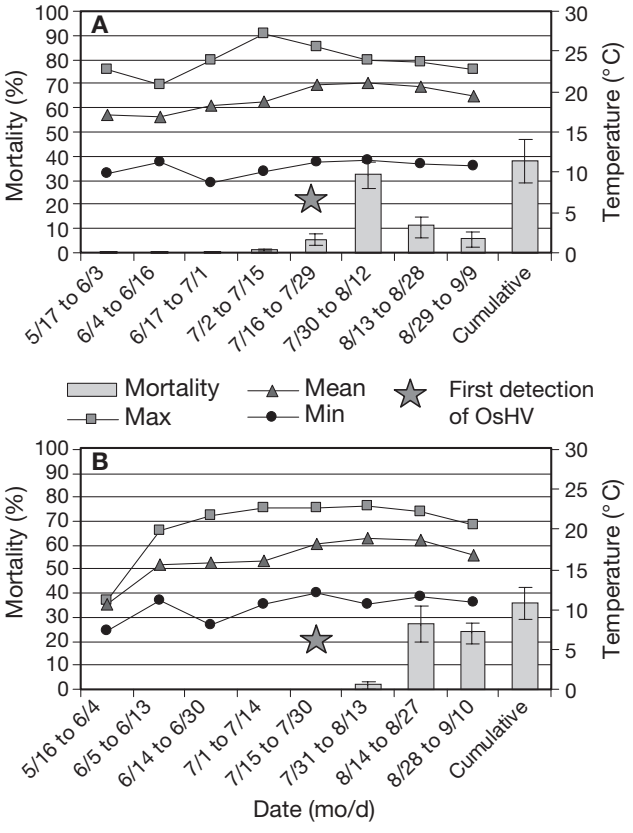

Fig. 3. *Crassostrea gigas.* Relationship between mortality, ostreid herpesvirus (OsHV) presence, and bimonthly temperature minima, means, and maxima experienced by oysters planted in (A) Inner and (B) Outer Tomales Bay, in 2003. (A, B) Mean temperature was similar between sites ($p > 0.05$) and predicted OsHV presence ($p < 0.005$); OsHV presence predicted mortality)$p = 0.01$)

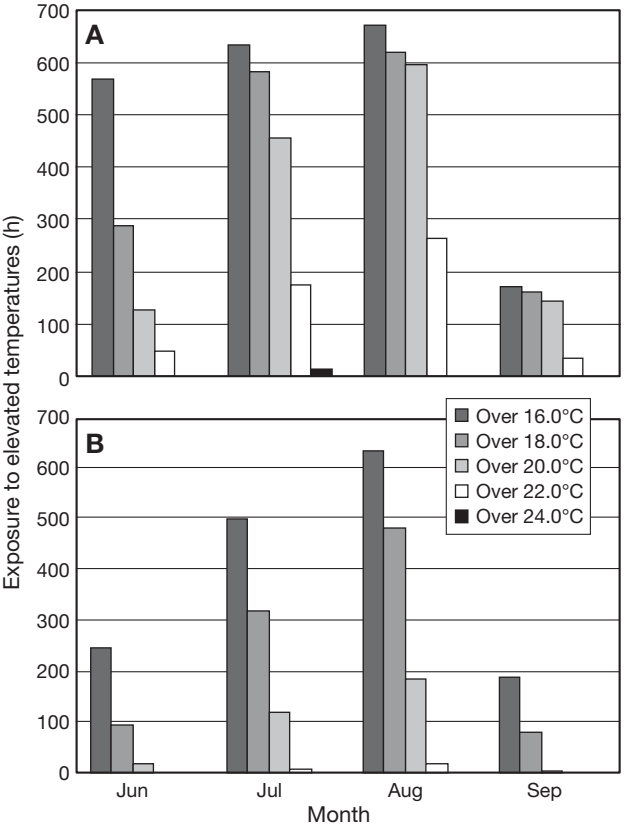

Fig. 4. *Crassostrea gigas.* Hours of exposure to temperatures above 16, 18, 20, 22, and 24°C experienced by oysters planted in the (A) Inner and (B) Outer Tomales Bay, in 2003. Oysters grown in the (A) Inner Bay were exposed to longer hours of elevated water temperatures at 18°C ($p < 0.01$), 20°C ($p < 0.05$), and 22°C ($p < 0.02$),but not 16°C ($p > 0.05$) or 24°C ($p > 0.05$), than those grown at the (B) Outer Bay site

Oyster herpesvirus, temperature exposure, and mortality

PCR results

Amplifiable nucleic acid was present in all samples as evidenced by amplification of SSU DNA or OsHV DNA (Table 4). No PCR evidence of OsHV DNA was observed from May to mid-July. OsHV DNA was detected at both Inner and Outer Bay sites in late July (July 29/30 sample dates), and OsHV DNA was present in all groups tested: WA-1, WA-2, and WA-3. In late July, all pools (6 for each stock or a total of 18 pools) at the Inner Bay site showed presence for OsHV DNA, while 3 of 6 WA-1, 2 of 6 WA-2, and 4 of 6 WA-3 pools contained OsHV DNA at the Outer Bay site. In mid-August (August 12/13 sample dates) all tissue pools WA-1, WA-2, and WA-3 from Inner and Outer Bay sites contained OsHV DNA.

The relationship between presence of OsHV, mortality, outplant site, and various environmental parameters can be explained by 2 logistic regression models (please refer back to Fig. 3). In the first model, OsHV presence was used as the response and temperature (mean, minimum, and maximum), salinity, and outplant site were used as predictor variables. Mean temperature was the only significant predictor of OsHV presence ($p < 0.005$). Temperature minimum ($p > 0.05$), temperature maximum ($p > 0.05$), mean salinity ($p >$

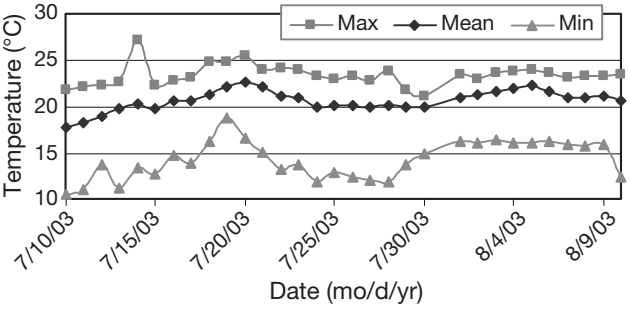

Fig. 5. Daily water temperature exposures experienced at the Inner Tomales Bay site between 10 July and 10 August 2003, depicted by minima, means, and maxima

Table 3. *Crassostrea gigas.* Correlations (C) between mortality and total exposure and degree hours per day at Inner and Outer Tomales Bay sites in 2003 and the combination of 2001, 2002, and 2003. (All data from 2001, 2002, and 2003 were compared in this treatment.) Two-tailed correlations between mortality and total exposure or degrees hours per day were tested using a Spearman Rank Correlation (SPSS 12.0). Total exposure refers to the total hours exposed to temperature from the beginning of the experiment until mortality occurred. Degree hours per day is total exposure divided by the number of days in the experiment until mortality occurred. ns: p-value is non-signficiant. na: Outer Bay temperatures did not reach 23, 24, or 25°C in 2003

Temperature (°C)	Inner Bay 2003	Outer Bay 2003	Inner Bay 2001, 2002, 2003	Outer Bay 2001, 2002, 2003
Total exposure				
16	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.802$)	ns	$p < 0.05$ ($C = 0.711$)
17	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.802$)	ns	ns
18	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.802$)	ns	ns
19	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.802$)	ns	ns
20	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.809$)	ns	ns
21	$p < 0.01$ ($C = 0.955$)	$p < 0.05$ ($C = 0.832$)	ns	ns
22	$p < 0.01$ ($C = 0.955$)	$p < 0.05$ ($C = 0.832$)	ns	ns
23	$p < 0.01$ ($C = 0.955$)	na	ns	ns
24	$p < 0.0001$ ($C = 0.984$)	na	$p < 0.05$ ($C = 0.658$)	ns
25	$p < 0.01$ ($C = 0.880$)	na	$p < 0.05$ ($C = 0.700$)	ns
Degree hours per day				
16	ns	$p < 0.05$ ($C = 0.802$)	ns	ns
17	$p < 0.05$ ($C = 0.820$)	$p < 0.05$ ($C = 0.802$)	ns	ns
18	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.802$)	ns	ns
19	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.802$)	ns	ns
20	$p < 0.01$ ($C = 0.941$)	ns	ns	ns
21	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.832$)	ns	ns
22	$p < 0.01$ ($C = 0.941$)	$p < 0.05$ ($C = 0.786$)	ns	ns
23	$p < 0.05$ ($C = 0.832$)	na	ns	ns
24	$p < 0.01$ ($C = 0.935$)	na	$p < 0.01$ ($C = 0.794$)	ns
25	ns	na	$p < 0.05$ ($C = 0.697$)	ns

Table 4. *Crassostrea gigas.* Sample schedule and results of PCR analysis of excised tissue samples taken from Pacific oysters in 2003. na: not applicable

Source	Site	Stock	Sample date(s) (mo/d)	No. tested	Proportion, % OsHV positive
Georgia Strait, WA	na[a]	WA-1	5/16	60[b]	0/6[c], na[d]
Hood Canal, WA	na[a]	WA-2	5/16	60[b]	0/6[c], na[d]
Hood Canal, WA	na[a]	WA-3	5/16	60[b]	0/6[c], na[d]
Yaquina Bay, OR	na[a]	OR-1	5/16	60[b]	0/6[c], na[d]
Yaquina Bay, OR	na[a]	OR-2	5/16	60[b]	0/6[c], na[d]
Tomales Bay, CA	Inner Bay	WA-1	6/3–15/7[e]	120	0/6[c], na[d]
		WA-2	6/3–7/15[e]	120	0/6[c], na[d]
		WA-3	6/3–7/15[e]	120	0/6[c], na[d]
	Outer Bay	WA-1	6/4–7/14[e]	120	0/6[c], na[d]
		WA-2	6/4–7/14[e]	120	0/6[c], na[d]
		WA-3	6/4–7/14[e]	120	0/6[c], na[d]
Tomales Bay, CA	Inner Bay	WA-1	7/29	30[f]	6/6[c], na[d]
		WA-2	7/29	30[f]	6/6[c], na[d]
		WA-3	7/29	30[f]	6/6[c], na[d]
	Outer Bay	WA-1	7/30	30[f]	3/6[c], na[d]
		WA-2	7/30	30[f]	2/6[c], na[d]
		WA-3	7/30	30[f]	4/6[c], na[d]
Tomales Bay, CA	Inner Bay	WA-1	8/12	30	6/6[c], 53.33 ± 28.87[c,g]
		WA-2	8/12	30	6/6[c], 66.67 ± 20.82[c,g]
		WA-3	8/12	30	6/6[c], 41.48 ± 2.57[c,g]
	Outer Bay	WA-1	8/13	30	6/6[c], 73.33 ± 11.55[c,g]
		WA-2	8/13	30	6/6[c], 50.00 ± 10.00[c,g]
		WA-3	8/13	30	6/6[c], 33.33 ± 25.17[c,g]

[a]Groups of oysters from each stock were sampled pre-outplant on 5/16/2003
[b]Pooled sample: 12 pools of 5 oysters were sampled per group for a total of 60 oysters
[c]All samples contained amplifiable DNA
[d]Prevalence of OsHV was not tested on this date
[e]Sample dates during this period were as follows for the Inner Bay: 6/3, 6/16, 7/1, and 7/15, and the Outer Bay: 6/4, 6/13, 6/30, and 7/14 with 30 oysters per group sampled on each date or a total of 120 oysters for the 4 dates
[f]Pooled sample: 6 pools of 5 oysters were sampled per group for a total of 30 oysters in each group per sample date
[g]Prevalence is listed as percent infected ±1 SD

0.05), and outplant site ($p > 0.05$) were not significant predictor variables. The second logistic regression model used mortality as the response variable and temperature (mean, minimum, and maximum), mean salinity, outplant site, and OsHV presence as the predictor variables. OsHV presence was the only statistically significant predictor of mortality ($p = 0.01$), while temperature minimum ($p > 0.05$), temperature maximum ($p > 0.05$), mean salinity ($p > 0.05$) and outplant site ($p > 0.05$) were not statistically significant. Additionally, only models using data for both sites were significant, models using only data from the Inner Bay (all variables listed above, $p > 0.05$) or the Outer Bay (all variables listed above, $p > 0.05$) were not significant.

Prevalence of OsHV DNA in tissue collected from individual oysters at both the Inner Bay and Outer Bay sites was quantified in mid-August (August 12/13 samples dates). Prevalence ranged from 20 to 90% at the Inner Bay site (average = 53.93 ± 20.91% [mean ± 1 SD]); at the Outer Bay prevalence ranged from 10 to 80% (52.22 ± 22.79%). Prevalence of OsHV DNA was high in all stocks and no difference was detected among groups ($p > 0.05$) or sites (Fig. 6; $p > 0.05$). When comparing prevalence and cumulative mortality on an individual bag basis, log-transformed prevalence (August 12/13 sample dates, as above) and cumulative mortality were significantly related using a linear model (Fig. 7; $p < 0.0001$).

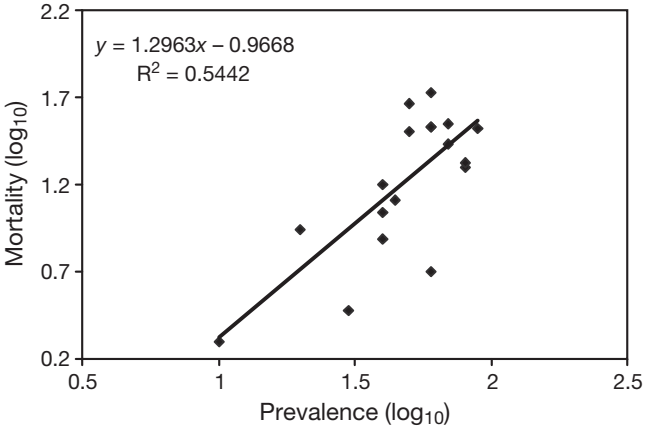

Fig. 7. *Crassostrea gigas.* Statistically significant relationship ($p < 0.0001$) between individual bag log-transformed mortality and individual log-transformed OsHV prevalence using data from Inner and Outer Tomales Bay, sites in 2003

Histology

Subsets of oysters planted in this experiment were examined by histology. Thirty oysters collected in June (before mortality or the detection of OsHV) and 35 oysters collected at the beginning of the mortality events on July 29 (when OsHV was first detected in this experiment) had normal tissue architecture. Abnormal tissue architecture and cellular changes were noted in the 12 oysters examined that tested positive for OsHV based on PCR analysis from the August 12/13 samplings. Most oysters had morphological changes in digestive tubule epithelia including cubodial metaplasia, tubule dilation and diapedesis; diapedesis was also noted at the mantle margin of some oysters. Additionally, diffuse hemocyte infiltration and lysed connective tissue were observed. Cowdry type A nuclear inclusions consistent with herpesviruses were not found in any of the oysters examined. However, nuclear changes including chromatin margination and pycnosis were observed. Swollen hemocytes with a small nucleus to cytoplasm ratio were noted circulating in the digestive gland, mantle, and connective tissue (Fig. 8).

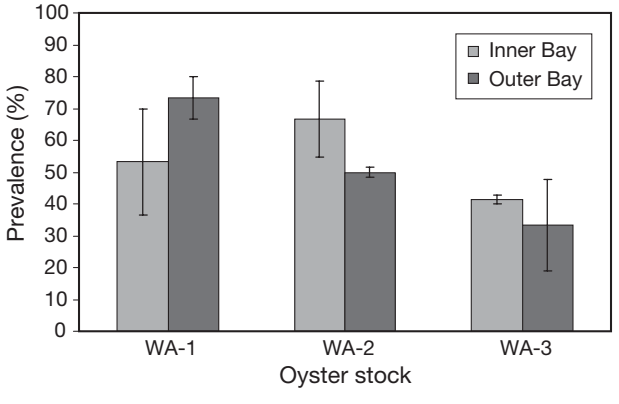

Fig. 6. *Crassostrea gigas.* Average OsHV prevalence ±1 SD detected in 3 stocks on August 12 at Inner Tomales Bay and August 13 at Outer Tomales Bay, in 2003. Statistical differences were not detected among stocks or between sites

Relationships between oyster size, growth, and mortality

Height (Fig. 9) and weight (data not shown) of oysters collected between May 16 and September 10 were correlated according to the allometric relationship weight = $0.1773\,\text{height}^{2.3812}$. Final shell height and weight of oysters grown at the Outer Bay site were greater than those grown at the Inner Bay site ($p = 0.005$ and $p < 0.0001$, respectively). A site by stock interaction occurred between sites in weight ($p < 0.0001$) and height ($p < 0.0001$). Each measurement followed the same general trend; OR-1 was similar to OR-2 < WA-2 which was similar to WA-1 < WA-3 (Fig. 10). This general size trend is the reverse of oyster mortality trends, where smaller oysters (groups OR-1 and OR-2) had the greatest mortality.

Growth rates were calculated using both shell height and weight. Growth rate (based on weight) differed

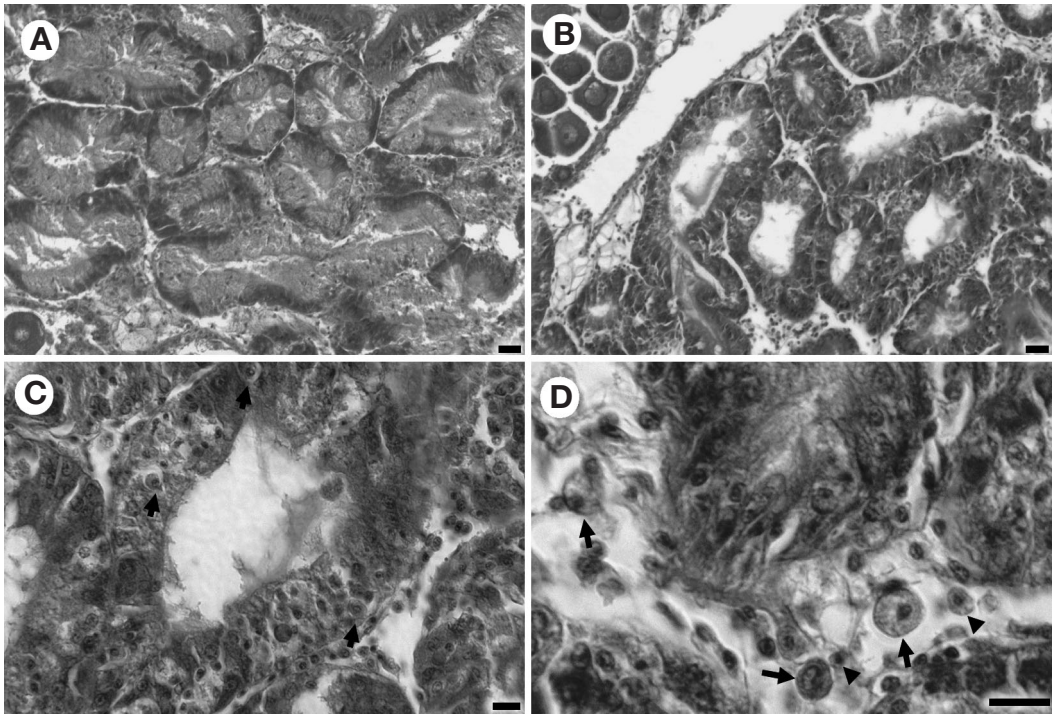

Fig. 8. *Crassostrea gigas.* Digestive gland and cellular architecture of oysters collected in Tomales Bay, in 2003 showing: (A) a normal digestive gland of an oyster collected at the beginning of the mortalities (July 29) (scale bar = 20 µm); (B) cuboidal metaplasia and digestive gland tubule dilation of an oyster collected during a mortality event (August 12) (scale bar = 20 µm); (C) hemocytes with marginated chromatin undergoing diapedesis (arrows), during a mortality event (August 12) (scale bar = 10 µm); (D) swollen hemocytes with a small nucleus to cytoplasm ratio (arrows) in comparison to normal hemocytes (arrowheads) (scale bar = 20 µm). Hemotoxylin and eosin

among stocks ($p < 0.0001$; Fig. 11), but not between sites ($p > 0.05$) and a site by group interaction did not occur ($p > 0.05$). Linear relationships between growth (both length and weight separately) and survivorship did not exist at the Inner Tomales Bay ($p > 0.05$) or Outer Tomales Bay site ($p > 0.05$).

DISCUSSION

In the current study (conducted in 2003), seed mortalities followed temperature extremes and OsHV detection at Inner Bay and Outer Bay sites. When seed oysters first began dying in Tomales Bay in 1993, mor-

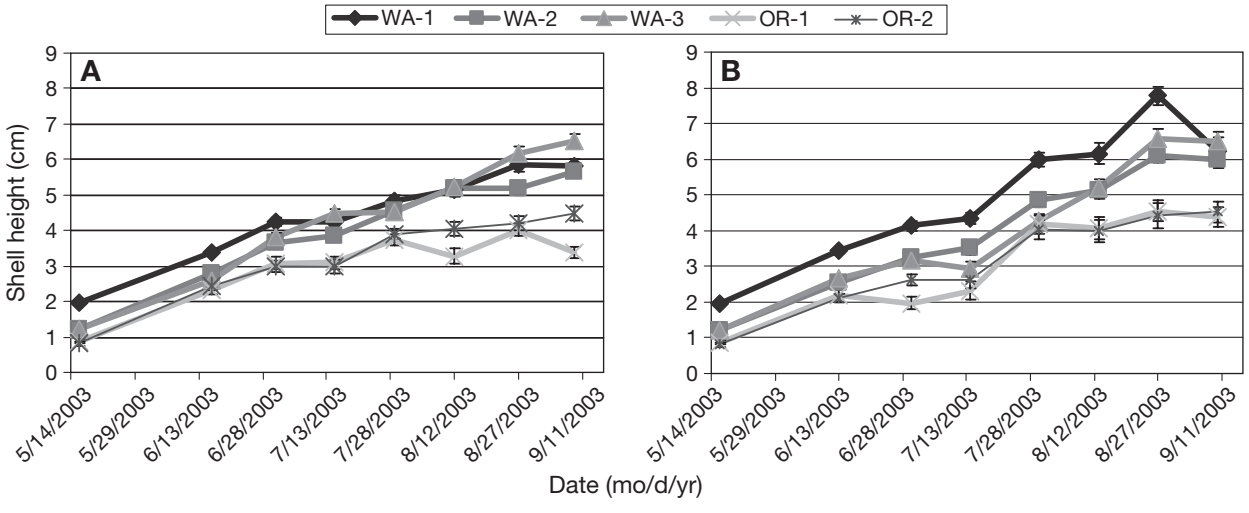

Fig. 9. *Crassostrea gigas.* Shell height (mean ± 1 SE) of stocks grown in (A) Inner and (B) Outer Tomales Bay, in 2003

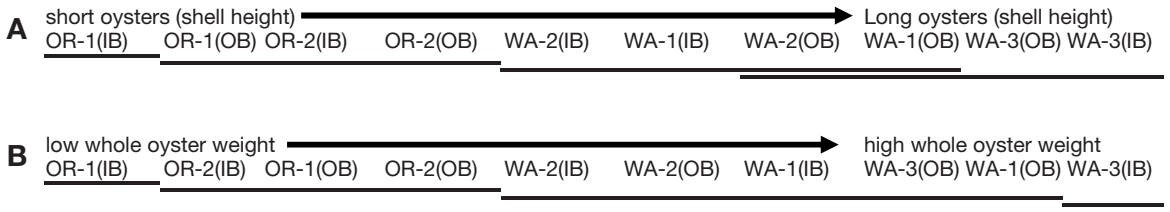

Fig. 10. *Crassostrea gigas.* Statistical differences ($p < 0.05$) among stock (A) height and (B) weight at the Inner Bay (IB) and Outer Bay (OB) sites in Tomales Bay, in 2003

tality was especially strong at the Outer Bay site (Cherr & Friedman 1998). In 1995, Outer and Central Bay sites experienced mortality in July followed by less severe mortality at the Inner Bay site (Cherr & Friedman 1998). Patterns of mortality changed in later years (2000, 2001, and 2002), when mortality was strong at the Inner Bay site and almost non-existent at the Outer Bay (C. S. Friedman unpubl. data). While sites affected by mortality have changed since 1993, a pattern associated with mortality has emerged: temperature extremes in excess of 24 to 25°C preceded mortalities in 1995, 2000, 2001, and 2002. In 2 of these years, 1995 and 2002, OsHV was detected in tissues sampled from oysters grown at the Inner Bay site (Friedman et al. 2005, C. S. Friedman unpubl. data), but the relationship between mortality, temperature exposure, and infection of oysters by OsHV was not studied.

These observations support previous research on Tomales Bay seed losses (Friedman et al. 2005, C. S. Friedman unpubl. data) and OsHV induced losses in larval oysters (Le Deuff et al. 1996). In an experiment using larval Pacific oysters, mortalities induced by OsHV were more rapid and severe in larvae held at 25 to 26°C than those held at 22 to 23°C (Le Deuff et al. 1996). Similarly, temperature is known to influence the onset and severity of several fish viral diseases (e.g. Getchell et al. 1998, Ahne et al. 2002, or Gilad et al. 2004). For example, both time to infection (Gilad et al. 2003, 2004) and cumulative mortality (Gilad et al. 2003) of koi *Cyprinus carpio koi* infected with koi herpesvirus are influenced by water temperature. Koi exposed to higher water temperatures (23 and 28°C) had a more rapid onset of high mortality (88.4 to 95.2%) than fish held at moderate (18°C and 85% mortality) and low temperatures (13°C and 0% mortality) (Gilad et al. 2003).

Although cumulative mortality was similar between sites, seed mortalities occurred more rapidly at the Inner Bay than those at the Outer Bay site. Losses at the Outer Bay occurred after temperatures exceeded 22°C, a temperature that is thought to be associated with incomplete virion production (Le Deuff et al. 1996). Le Deuff et al. (1996) hypothesized that OsHV may require temperatures in excess of 25°C to replicate. Temperatures less than 25°C would then yield one of two stages where viral particles are rare (true latent stage) or absent (abortive stage), but structural changes could occur in the infected cell nucleus. Extrachromosomal latent herpesvirus infections may be induced to replicate leading to a productive viral infection, as is typical in viruses of the family *Herpesviridae* such as OsHV (Davison et al. 2002, 2005). The exact mechanism of viral induction in herpesviruses is unknown, but may be dependent on the presence of stimuli such as temperature or light, the virus-host combination, and or the physiology of the infected cell (Champoux 2004).

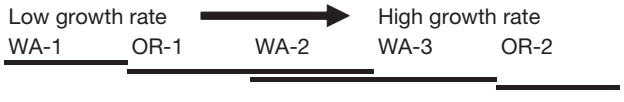

Fig. 11. *Crassostrea gigas.* Statistical differences ($p < 0.05$) among growth rates of oysters in stocks grown in Tomales Bay, in 2003

OsHV DNA was first detected 2 wk after the first temperature maximum in excess of 25°C at the Inner Bay site. Based on this evidence, it is hypothesized that temperature maxima ≥25°C may induce OsHV viral replication. Given that adult bivalves of many species are infected by OsHV, e.g. *Ostrea angasi* (Hine & Thorne 1997), *Crassostrea gigas* (Arzul et al. 2002), and *Pecten maximus* (Arzul et al. 2001c), it is hypothesized that adult *C. gigas* or other bivalves (7 species are grown in Tomales Bay, including: *O. edulis*, *C. sikamea*, *Venerupis phillipinarum*, *C. virginica*, *Mytilus galloprovincialis*, and *O. conchaphila*) grown in Tomales Bay harbor latent OsHV infections. Therefore, testing of OsHV infection in all bivalve species cultured in Tomales Bay is needed. When water temperatures exceed 24 to 25°C, viral replication may occur in adult bivalves harboring latent infections allowing transmission of the virus to seed oysters followed by mortalities such as those documented here.

Despite differences in temperature exposure between the Inner and Outer Bay sites, OsHV DNA was detected in oyster tissue on the same day at both Inner and Outer Bay sites, and a higher proportion of pools at the Inner Bay site contained OsHV DNA. It is unknown whether oysters grown at the Outer Bay site were infected with OsHV in 2001 and 2002, when mortali-

ties were low at this site but high at the Inner Bay site. Temperatures at the Outer Bay site in 2001, 2002, and 2003 have been consistently cooler (average, maximum, and overall thermal exposure) than those at the Inner Bay site (C. S. Friedman unpubl. data). Temperature extremes in excess of 22°C occurred more frequently at the Inner Bay site in 2003, than those experienced in 2001 and 2002, particularly in July and August during OsHV induction and oyster mortalities (Table 5). Therefore, it is hypothesized that increased thermal exposure (over 2001 and 2002) at the Inner Bay site in 2003 led to (1) faster viral replication and earlier mortalities at the Inner Bay site (versus the delayed mortalities coupled with lower temperature at the Outer Bay site), and (2) an increase in the number of infectious virions available to spread from the Inner Bay site to the Outer Bay site leading to the detection of OsHV DNA and subsequent mortalities.

Table 5. *Crassostrea gigas.* Monthly mortality and temperature exposure (expressed as hours in excess of 20, 22, and 24°C) experienced by oysters grown in Tomales Bay, CA, in 2001, 2002, and 2003

Site Year	Month	Mortality	Hours exposed to elevated temperatures in excess of: 20°C	22°C	24°C
Inner Bay					
2001	May		139	22	4
	June	X	239	59	13
	July[a]		158	63	3
	August		295	53	0
2002	June	X[c]	68	1	0
	July		379	62	4
	August	X[d]	307	157	1
	September		186	6	0
2003	June		127	47	0
	July		456	176	12
	August	X	594	265	0
	September		144	154	0
Outer Bay					
2001					
	May		36	11	0
	June[b]				
	July		34	4	0
	August		44	8	1
2002	June		0	0	0
	July		28	0	0
	August		0	0	0
	September		2	0	0
2003	June		17	0	0
	July		120	9	0
	August	X	185	20	0
	September		3	0	0

[a]No data was collected from July 10–24
[b]No data was collected during June
[c]Low mortality
[d]High mortality

Despite plausible differences in replication rates, oysters collected in mid-August (August 12/13 sample dates), when mortality was low at the Outer Bay and high at the Inner Bay, had similar OsHV prevalences. Using primers from the C fragment of OsHV-1 (C9/C10), Barbosa-Solomieu et al. (2004) described percentages of OsHV prevalence that are similar to those found in this study. In *Crassostrea gigas* and *Ostrea edulis* that were deemed apparently healthy and of intermediate health based on microscopic changes using paraffin histology, 69.6 and 32.2%, respectively, were infected with OsHV (Barbosa-Solomieu et al. 2004). While the use of conventional PCR in this study and the one conducted by Barbosa-Solomieu et al. (2004) adequately characterized OsHV prevalence, the intensity of infection (viral loads) and characterization of the presence of unproductive (abortive or latent) or productive infections were not possible. Productive infections may lead to mortality in the host species, but apparently healthy individuals, not characterized by cellular changes associated with OsHV, that test positive for OsHV may be shedding the virus (Barbosa-Solomieu et al. 2004) as occurs in herpesviruses infecting humans (e.g. Kosaki et al. 2003 and Sacks et al. 2004) and other animals (e.g. Six et al. 2001) with productive (but often asymptomatic) infections. Viral load may characterize numerical differences between productive, mortality-causing infections and infections not leading to mortality, both non-productive and productive (as described above), and may also provide an explanation for early onset of mortality at higher temperatures. For example, viral load in koi infected by koi herpesvirus was higher in fish held at higher temperatures where fish underwent an earlier onset of mortality, and lower for fish held at lower temperatures that did not experience mortality (Gilad et al. 2004).

The viral load or the number of virions in each oyster may facilitate OsHV-induced seed mortalities. OsHV pathogenesis in seed oysters is unknown, although many papers have described tissue and cellular changes in OsHV-positive animals. Many of the abnormal cellular and tissue characteristics described in this study, including dilation of digestive gland tubules, cells undergoing diapedesis, inflammation, and nuclear changes of cells, are consistent with past Tomales Bay mortalities (C. S. Friedman unpubl. obs.) and previous histological observations of OsHV infections in Tomales Bay (Friedman et al. 2005) and France (Renault et al. 2000a). In conclusion, OsHV and elevated temperatures are a likely cause of summer seed losses in Tomales Bay, California. Further studies to quantify the viral load needed for a productive, mortality causing OsHV infection and understanding the pathogenesis of OsHV in seed

oysters are needed.

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