Examining the roles of environment, host, and pathogen in the host-pathogen relationship between the oyster herpesvirus and the Pacific oyster

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Presentation Outline

- **Background**
  - THE PATHOGEN: OsHV in the USA
  - THE HOST: *Crassostrea gigas*, the Pacific oyster
  - THE ENVIRONMENT: Tomales Bay (TB), California

- **Summer seed mortality (SSM) and OsHV in TB**
  - Field surveys 2000-2003
  - Multispecies survey 2003

- **OsHV transmission in larvae**
  - Basic transmission from seed to larvae
  - Gene identification
OsHV in the USA 1

- The first herpesvirus detected in bivalves was described in the 1970’s by Farley et al. 1972 in Maine (East Coast)
- Currently unknown how similar this virus is to the characterized OsHV-1
- No other herpes-like viruses were described in the USA until the early 2000’s when OsHV was detected in Tomales and Drakes Bays using an OsHV-specific cPCR (West Coast)
- Sequence results indicate a virus similar to OsHV-1
OsHV in the USA II

- cPCR survey by Friedman et al. 2005
- Unpub. cPCR and/or histology R. Elston
- Unpub. cPCR C. Burge/C. Friedman

*Species tested: primarily Pacifics but also Kumamotos and Eastern oysters*
THE HOST: the Pacific oyster

- Imported from Japan to embayments along the US West Coast and globally
- Hardy and fast growing species replaced the West coast native oyster Ostrea lurida
- Naturalized in some areas of the US West Coast; other areas depend on oyster hatcheries
Short pulses of mortality of juvenile Pacific oysters have occurred nearly annually since 1993 (up to 90%).

In 1993-1994, cumulative losses of 5 farms approached 50-65% in 3 months (one summer).

Previous to 1993, losses over an 18 month culture cycle were typically 8-35%.

Other cultured bivalves appear to be unaffected.

Sentinel field study of 1995 reported mortalities correlated with warm temperatures (up to 25 C) & phytoplankton blooms.

Initially no disease agent was identified with mortalities; OsHV was first detected in 2002 & has been detected as early as 1995.
Host X Environment in Tomales Bay

Cumulative Percent Mortality

- **Mortality**
- **Average Temp**
- **Maximum Temp**

**Percent Mortality**

- May 15-31
- June 1-15
- June 16-30
- July 1-15
- July 16-31
- Aug 1-15
- Aug 16-31
- Sept 1-15
- Sept 16-30

**Degrees C**

- 0
- 5
- 10
- 15
- 20
- 25
- 30

**Average Temp** and **Maximum Temp** show a relatively stable trend throughout the months. The **Mortality** line indicates significant spikes in June and July, suggesting a correlation with temperature changes.
THE ENVIRONMENT: Tomales Bay
Basic research questions

Field Based Research Questions

★ Can we improve seed survival in TB?
★ Is OsHV or oyster health associated with SSM in TB?
★ Are other stressors involved in mortalities?
★ Are other species in Tomales Bay infected with OsHV?

Lab Based Research Questions

★ Is OsHV detected in Tomales Bay infectious?
★ What is the host response to OsHV?
Methods: Sentinel Field Studies 2000-2003

- Cohorts of 3-5 stocks per year
  - Low and high performing family lines, and diploid and triploid hatchery stocks

- Environmental monitoring: temperature, salinity, & phytoplankton

- Health status, growth, and survival were monitored
  - Histology
  - OsHV-specific cPCR in 2003

Burge et al. 2006, 2007
Can we improve seed survival in TB?

- Plant time (spring versus fall $p<0.05$) in 2000-2001 and seed source greatly affected survival in 2000-2003 ($p<0.0001$)

- Smaller oysters are preferentially affected, in some years the fastest growing oysters had the highest mortality rate
Mortality and seed source: 2000-2001

Mortality significantly greater at the Inner Bay site (p<0.0001)  
a>b>c>d (p<0.05-p<0.0001)
Mortality significantly greater in the spring stocks of WA-1 and OR-2 (p<0.0001)
Indicates improved seed survival is possible
Data from small-scale “selective” breeding expts (2008-2009) confer
Results: Sentinel Field Studies 2000-03

- Is OsHV or oyster health associated with SSM in TB?
  - Histological signs of any abnormalities rare
  - First detected OsHV in 2002 with cPCR
  - Associated OsHV presence with mortality 2003 with cPCR

- Is mortality associated with abiotic or biotic stressors?
  - Temperature: elevated means and maximums related to mortality (dependent on year)
  - Salinity (2000-2003) & phytoplankton (2000-2001) unrelated to mortality (p>0.05)
Using data from both sites, mean temperature predicted OsHV presence (p < 0.005), and OsHV presence predicted mortality (p<0.01)
OsHV prevalence: 2003

Is OsHV prevalence associated with differential mortality?

- Prevalence only measures the percent of animals with viral DNA
- Viral load and infection status may be different between sites
- Asymptomatic animals may be in the processing of shedding the virus

ANOVA: p>0.05; prevalence is not different between sites or families
Temperature and mortality in TB

- Mortality events stronger at the Inner Bay site where greater temperature maximums were recorded.
- Temperature may be related to rate of viral replication.
- Mortalities at the Inner Bay site from 2001, 2002, and 2003 significantly correlated (p<0.05) with total exposure and degree hours >24 & 25°C and not temps between 16-23 °C (p>0.05).
How are oysters being infected?

- Uninfected seed are outplanted each year
  - OsHV not detected in hatcheries to date in the US
- Mortality occurs only after temperature extremes
  - Temperature extremes not considered to be lethal
  - Temperature (>25C) has been related to larval mortalities & may trigger OsHV viral replication
- Adult oysters in the bay may have latent infections
- Temperature may trigger viral replication of latent OsHV infections leading to OsHV transmission to naïve individuals
Multi-species Survey 2003

Are other species in Tomales Bay infected with OsHV?

Test multiple species collected in TB with cPCR
Develop OsHV-specific qPCR assay
Test multiple species collected in TB with qPCR
Methods: Multi-species Survey 2003

- In late summer of 2003, multiple species of bivalves were collected.
- Pacific oysters were also collected from Drakes Estero.
- Tested with cPCR and qPCR in conjunction with histology.

Burge et al. in review
OsHV prevalence using cPCR

Species

- Mediterranean mussels
- Eastern oysters*
- European flat oysters*
- Kumamoto oysters*
- Manila clams
- Olympia oysters
- Pacific oyster*
- Pacific oyster (DE)*

* Sequence confirmation

Prevalence

- n=60
- n=60
- n=14
- n=60
- n=54
- n=38
- n=60
- n=36

Species

- Mediterranean mussels
- Eastern oysters*
- European flat oysters*
- Kumamoto oysters*
- Manila clams
- Olympia oysters
- Pacific oyster*
- Pacific oyster (DE)*
OsHV-specific qPCR: A fragment

- Cycle threshold
- OsHV plasmid copies per reaction (log scale)

$\text{r}^2 = 0.997$

Reaction efficiency = 95.5%

Limit of detection: 1 copy
OsHV copy number in select species from TB

Species

<table>
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<tr>
<th>Pacific oysters</th>
<th>European Flat oysters</th>
<th>* Manila Clams</th>
<th>Kumamoto oysters</th>
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<td>b</td>
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Copies of OsHV DNA per ng of gDNA

a>b; p< 0.0001

*Sequence confirmation

qPCR also detected OsHV in 13 additional individuals
OsHV detected in multiple species

- Low copy number may infer latent infections
- No evidence of OsHV infection with light microscopy
- Neither cPCR or qPCR confirm infection
- PCR positive individuals may act as reservoirs
- Methods to confirm OsHV such as Electron Microscopy or *in situ* hybridization are less sensitive and more cumbersome
- A rapid, sensitive molecular method that detects only active viruses may be helpful
Basic research questions

Field Based Research Questions

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- Is OsHV or oyster health associated with SSM in TB?
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Lab Based Research Questions

- Is OsHV detected in Tomales Bay infectious?
- What is the host response to OsHV?
OsHV experimental transmission

- In France, OsHV-1 has been transmitted between and among multiple larval bivalve species.
- OsHV transmission from infected seed to naïve seed was recently completed.
- OsHV transmission from infected seed to naïve larvae has not been previously described.
Part I: Research Questions/Objectives

- Is OsHV detected in California infectious?
  - Use OsHV infected tissue from TB to attempt experimental transmission from infected seed to naïve larvae

- Can we see differences in OsHV quantification using primer sets based on different parts of the genome?
  - Quantify viral load by comparing DNA based OsHV-specific qPCR designed on two genes

- Can we detect/quantify active virus infection?
  - Quantify active virus infection using a RT qPCR based on the OsHV DNA polymerase gene
Homogenates were created from uninfected seed (Washington State) or infected seed oysters (Tomales Bay) stored at -80°C.

Tissue was homogenized in sterile seawater, filtered through a 0.22 µm filter, and exposed to oyster larvae held at 25°C (Arzul et al. 2001).
Experimental Methods 2

- 7 day old larvae (100/mL)
  - Trial 1 (3X each)
    - Controls:
      - Filtered homogenate (WA)
      - Seawater
    - Exposed:
      - Filtered homogenate (TB)
  - Trial 2 (9X each)
    - Control:
      - Filtered homogenate (WA)
    - Exposed:
      - Filtered homogenate (TB)
Experimental Methods 3

- **Trial 1:**
  - Survival was enumerated every other day from each flask by taking three 1 mL samples which were used for DNA and RNA assays (< 50 larvae)

- **Trial 2:**
  - Repeated Trial 1 except survival was enumerated on Day 1, 4 and 7 and whole flasks were sampled (n=3 per day) for Electron Microscopy and increased starting material for DNA and RNA assays

- **Daily care:** water changes and feeding (T. ISO)
Basic Molecular Methods

- To quantify viral load: qPCR
  - A primers: a protein of unknown function
    - Reaction efficiency: 93.2% ± 0.56% (+ SE)
    - $R^2: 0.994 ± 0.00095%$
  - DNA poly primers: DNA polymerase gene
    - Reaction efficiency: 98.5 ± 1.89% (+ SE)
    - $R^2: 0.993 ± 0.001%$

- To quantify viral replication (active virus): RT qPCR
  - DNA poly primers: DNA polymerase gene
Results 1: Larval survival curves

- Seawater Control
- Homogenate Control
- Homogenate Treatment
Results 2: OsHV viral load

Primer Quantification:
No significant differences between A primers and DNA polymerase primers (p > 0.05)
Results 3: Quantifying active virus

![Graph showing active virus copy number per ng of TOTAL RNA over Experiment Days.]

- **DNA Pol**
  - Active virus copy # per ng of TOTAL RNA
  - Days 0 to 9
  - Days 0 to 9

- **a>b; p< 0.0001**

**Legend:**
- Green line with markers indicating data points and error bars for **DNA Pol**.
- Days 0 to 9 are labeled on the x-axis.
- Active virus copy number ranges are labeled on the y-axis.

**Notes:**
- The graph illustrates a significant difference in active virus copy number over time, with specific p-values indicating statistical significance.
Results 4: Trial 2, EM 1
Results 5: Trial 2, EM 2
Part II: Research Questions/Objectives

★ What is the host response to OsHV?
Identify genes involved in host defense
Gene identification

- RNA pools were created from RNA extracted from day 1 samples of exposed and control groups (Trial 2)

- SOLiD sequencing was used to identify genes involved in host response and virus infection
SOLiD Sequencing Methods

- Next generation sequencing approach where all mRNA transcripts in a given sample are sequenced in short reads of ~50 bp
- Sequences can be aligned (matched) giving longer reads of sequences either against known databases: GenBank and for oysters Sigenae (public database of oyster contigs)
- A library of larvae exposed to OsHV has been sequenced (control library to come)

**1 Assembly report**

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Sigenae assembly report
SOLiD Sequencing Results 1: Host

Binned Immune Genes: Hits 1% or greater

Gene Function based on defined function of Sigenae Contigs using Go terms & binned using CataGOrizer

- Metabolism (general, protein, carbohydrate)
- Stress response
- Mitochondrion
- Apoptosis/regulation of apoptosis
- Catabolism
- Endocytosis
- Intracellular signaling cascade
- Cell adhesion
- Death
- Response to external stimulus
- Other

[Diagram showing percentage distribution of gene functions]
Most OsHV gene function is unknown, including open reading frames (ORFs) with large number of hits.

Genes thought to be anti-apoptosis (IAPs) were not present in high numbers.
Discussion of Gene Identification

- A SOLiD library of genes expressed in the controls will be important to correctly identify genes expressed in response to OsHV
- The SOLiD results provided new sequence information about OsHV detected in California
In the bigger picture

Temperature leads to transmission & mortality
Careful selection of oyster stocks
Transmission from infected seed to larvae
Improved diagnostic methods
Method to detect active infection
Identification of genes
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Tomales & Drakes Bays
Questions?

Charlene Burge
No major phytoplankton blooms at the Inner Bay during mortalities in June; Additionally, only low levels of the potentially toxic *Akashiwo sanguinea* were recorded.