Inter-annual variation in the reproductive pattern of Manila clam *Ruditapes philippinarum* and impacts of *Perkinsus olseni* infection on the reproduction observed from the west coast of Korea

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Ruditapes philippinarum, the Manila clam

- Scientific name: *Tapes, Ruditapes, Venerupis philippinarum*

- Common name: Manila clam, short neck, little neck clam, Japanese little neck clam

- Introduced to the west coast of USA and to the European countries including Portugal, Spain, France and Italy

- Considered to be one of the most important species in world shellfish aquaculture industry
Ruditapes philippinarum in Pacific Asia Region
The Manila clam is endemic species to the costal Yellow sea and commonly cultured in Korea, China and Japan.

The Manila clam *Ruditapes philippinarum* is one of the most important marine shellfish resources supporting Korean fisheries industry.

*R. philippinarum* is intensively cultured on muddy or sandy tidal-flats along the coastal Yellow Sea and on the southern coast of Korea.
Clam (Ruditapes philippinarum) culture bed

- Clam seeds are naturally abundant in the beds
- Clam seeds are collected from spat-fall ground
- Seeds are also produced from hatchery
- 5-15mm seeds are sowed on commercial culture grounds
- After 2-3 years of grow-out period, they are harvested manually by local clam growers cooperative
- Whole sale price of 1.5-2.5 dollars and retail price of 3-4 dollars
Problems Identified in Korean Clam Industry

- Recurring mass mortalities of the clams in the clam beds in early spring or late summer.
- Poor condition of the clams; poor growth and reproduction.
- Pathogenic organisms such as *Perkinsus olseni* and *Vibrio tapetis*-like bacteria have been identified from gaping clams as well as physiologically poor clams.
Quantification of Reproductive Effort (RE)

- Understanding life histories and successful management
- Problems involved in assessment of RE in marine bivalves; gonads are integral part of the visceral mass in most bivalves

How to estimate RE of marine bivalves?

- Histological preparations
- Determining the difference in body weight
- Counting or weighing the eggs
  - Spawning is often incomplete
  - Occurs continuously
  - Semi-quantitative

- Immunological methods (Enzyme-linked immunosorbent assay, ELISA)
  - Rapid
  - Low cost
  - High sensitivity
What is *Perkinsus*?

- Perkinsosis is a protozoan parasitic shellfish disease occurring in some commercially important shellfish including oysters and clams.

- Responsible for the mass mortalities of the carpet shell clams *Ruditapes decussatus* in Europe and the eastern oyster *Crassostrea virginica*.

- Heavy infection with *Perkinsus* retards gonad maturation, spawning frequency and the reproductive effort.

- Difficulties involved in the study of impacts of *Perkinsus* infection on bivalve reproduction?

- Classified by the OIE as a disease that warrants notification.
Life Cycle of *Perkinsus olseni*

- **Proliferative stage**
- **Infectious stage**
- **Growth stage**
- **Vegetative multiplication stage**
- New host
- Zoospore
- Hypnospore
- Trophozoite
- Low oxygen
- Host tissue
- Sea water
- Zoospore
- New host
Microscopic appearance of *Perkinsus* in the tissue

- The nodule on the foot muscle
- Gill
- Muscle
- Gonad
Perkinsus olseni infection

• *Perkinsus olseni* has been identified from clams on the coastal Yellow sea and the southern coasts of Korea.

• Heavily infected clams often observed harmful effects such as slow growth, poor condition and low fecundity.

• Perkinsosis is often associated with mass mortality and subsequent decline in cultured and wild shellfish populations.
Objective

• To monitor spatio-temporal variation in Manila clam conditions on the west coast of Korea

  ➢ Annual reproductive cycle
  ➢ Reproductive Effort
  ➢ Perkinsus olseni body burden
Materials and Methods
Sampling site

✓ Hwangdo:
Commercial clam bed, silty-mud sediment

✓ Padori
Commercial clam bed, subtidal, silty-mud sediment

✓ Sampling period:
2007.1-2010.12 (48 months)

✓ Distance:
Padori-Hwangdo (25km)
Sampling site

- **Hwangdo**: Commercial clam bed, silty-mud sediment
- **Padori**: Commercial clam bed, subtidal, silty-mud sediment
- **Sampling period**: 2007.1-2010.12 (48 months)
- **Distance**: Padori-Hwangdo (25km)
Steps involved in the analysis of clam in this study

- **Clam Tissue**
  - Lyophilizing & Homogenizing Tissues
  - 1.5 mm Thick Cross Section
  - Histological Preparation
  - Identifying Sex and Gonad Maturation
  - Histopathological observation *Perkinsus* & trematode

- **Condition Index (CI)**
  - \( \text{CI} = \frac{\text{TWWT}}{\text{SDW}} \)

- **Dried Shell for Condition Index**

- **Homogenized Tissues**

- **Reproductive Effort by ELISA**

- **Gill or whole body**

- **Identifying Sex and Gonad Maturation**

- **Histopathological observation *Perkinsus* & trematode**

- **Perkinsus infection using RFTM/2M NaOH method**
Ray’s fluid thioglycollate medium assay (RFTM)

1. Gill or tissues were excised and incubate at room temperature in fluid thioglycollate medium (FTM) for 7 days at dark.

2. After FTM culture, the tissues digested in 2M NaOH.

3. All tissues were digested, add PBS in the conical tube till 5ml.

4. Number of *Perkinsus hypnospore* cells was counted under microscope using a hemocytometer.

5. *Perkinsus* infection intensity = hypnospores/g gill or tissue wet weight.
Histological observation

Preparation of the sample

Fixation

1.5 mm thick cross section of the body tissue
Davidson’s fixative
70% alcohol

Embedding

Automatic Tissue processor
Paraffin bath & Cold plate

Sectioning

6 µm from paraffin block

Staining

Hematoxylin & eosin

Mounting

Digestive gland
Mantle
Gill
Gonad
Digestive tract
Foot
Classification of reproductive stage of the Manila clam, *Ruditapes philippinarum* (Drummond et al, 2006)

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<th>Reproductive stage</th>
<th>Scale</th>
<th>Description</th>
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<td>Resting</td>
<td>0</td>
<td>Gonad follicle compose of connective tissue. The follicle is empty, oogonia cannot be observed.</td>
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<td>Early developing</td>
<td>1</td>
<td>Gonad proliferation initiates; increasing number of oocytes at follicular wall, no free oocyte in the follicles.</td>
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<td>Late developing</td>
<td>2</td>
<td>Free oocytes in the lumen but most oocytes attach on the follicular walls.</td>
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<td>Ripe</td>
<td>3</td>
<td>Gonad filling large surface area, oogenesis follicle full with polygonal configuration oocytes.</td>
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<td>Partially spawning</td>
<td>4</td>
<td>Numbers of free oocytes in lumen are decrease, empty space in follicle can be observed.</td>
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<tr>
<td>Spent</td>
<td>5</td>
<td>Follicles appear broken, scatter and relatively empty, only residual oocytes numerous numbers of phagocytes.</td>
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Gonad development of female *R. philippinarum*

**Early developing stage**

**Late developing stage**

**Ripe stage**

**Partially spawning stage**

**Spent stage**

**Resting stage**
Quntification of clam reproductive effort using Enzyme-Linked Immunosorbent Assay (ELISA)

Antigen

Blocking

Washing

Primary antibody
(Rabbit anti-clam egg specific antibody)

Washing

Secondary antibody
(Goat anti-rabbit IgG)

Washing

OD at 405 nm

Substrate
Quantity of RE in each clam = quantity of egg protein estimated from ELISA * 2.44

Egg protein = total egg weight x 0.4

Gonadosomatic index (GSI)
\[ GSI = \frac{\text{Total egg dry weight}}{\text{Total tissue dry weight}} \]

Fecundity
\[ \text{Fecundity} = \frac{\text{Total egg dry weight (g)}}{0.000022 \text{ g}} \]
Results
Water temperature

Water temperature (°C)

J F M A M J J A S O N D
2007 2008 2009 2010 Mean

Padori

<국립해양조사원; http://www.khoa.go.kr>
Water temperature

Hwangdo

Water temperature (°C)

0 5 10 15 20 25 30

J F M A M J J A S O N D

2007 2008 2009 2010 Mean

Water temperature

<국립해양조사원; http://www.khoa.go.kr>
# Field mortality

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Annual variation of tissue wet weight

Tissue Wet Weight (TWWT, g)

2007 2008 2009 2010

Padori

Hwangdo
Annual variation of condition index

Condition Index (CI)

Padori

Hwangdo
Perkinsus infection intensity

Perkinsus cell / g tissue weight

Padori

Hwangdo
## Perkinsus infection prevalence

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Perkinsus infection intensity (Padori)

Perkinsus cell / g tissue weight

Wi: Dec-Feb
Sp: Mar-May
Su: Jun-Aug
Au: Sep-Nov
Perkinsus infection intensity (Hwangdo)

Perkinsus cell / g tissue weight

Wi: Dec-Feb  
Sp: Mar-May  
Su: Jun-Aug  
Au: Sep-Nov

0 1,000,000 2,000,000 3,000,000 4,000,000 5,000,000 6,000,000

Wi  Sp  Su  Au  Wi  Sp  Su  Au  Wi  Sp  Su  Au  Wi

2007  2008  2009  2010
Annual reproductive cycle (Padori)
Annual reproductive cycle (Hwangdo)
Annual variation of Reproductive effort

Gonadosomatic Index (GSI, %)

Perkinsus infection

Condition index
## Spawning peak

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Note: The stars represent the months during which the spawning peak occurs.
Inter-annual variation in spawning season?

Inter-annual variation in *Perkinsus* infection?

Correlation between inter-annual variation in *Perkinsus olsenii* infection intensity and the reproductive effort?

Inter-annual variation in the water temperature and *P. olsenii* infection intensity?
Four years of the monitoring indicated that the spawning duration and the frequency varied year to year in the high infection area (Hwangdo).

*P. olseni* infection intensity also varied yearly.

GSI recorded in June 2010 (17%, prior to spawning) was significantly higher than the GSI measured in 2007, 2008 and 2009 (8-11%) in Hwangdo.

On the other hands, clams from Padori showed stable pattern of the GSI, ranging from 13-14% (prior to spawning) and low level of *P. olseni* infection intensity and prevalence.
Thank you for your attention!

I would like to thank everybody that participated in this project!