AQUACULTURE AND PATHOBIOLOGY OF CRUSTACEAN AND OTHER SPECIES

PROCEEDINGS OF THE THIRTY-SECOND U.S. JAPAN SYMPOSIUM ON AQUACULTURE

Davis and Santa Barbara, California U.S.A.
17-18th and 20th November 2003

Edited by
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Eileen McVey, and Melanie Caesar

Under the U.S.- Japan Cooperative Program in Natural Resource (UJNR)

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Contents

Summary of the Symposium On *"Aquaculture and Pathobiology of Crustacean and Other Species"* held in conjunction with the 32nd UJNR Aquaculture Panel Meeting in Davis and Santa Barbara, California U.S.A. 17-18th and 20th November 2003

The second year of the sixth five-year plan for scientific exchange focused on crustacean disease and pathology. The keynote addresses given the co-chairs included the importance of a more coordinated and more cohesive approach to the management of coastal resources and aquaculture following the five-year plan. Emerging issues such as sustainability, coastal zone management, and impacts on the environment create a more complex challenge for aquaculture research decisions that must be made at the highest levels of government. This type of international exchange can address those issues. Disease identification and control is essential in aquaculture and is a global management issue. In October 2003 the first case of the carp Herpes virus was detected in Japan. Dr. Sakai explained that this outbreak prevented key members of the Japanese research team from attending the 32nd meeting and personally thanked Professor Hedrick of UC Davis for providing valuable information in dealing with this disease. Since disease outbreaks such as this affect aquaculture globally this symposium was especially important for the exchange of research results.

Dr. Donald Lightner of the University of Arizona opened the symposium with an overview of the impact of four viruses affecting shrimp aquaculture and the resulting changes that have been made globally by shrimp farmers moving from wild captive stock to pathogen free broodstock. Additional presentations discussed disease prevention through disinfection, problems with breeding for disease resistance, establishing industry protocols, keeping published data up-to-date, control of live shipment and using non-compliance penalties to further control the spread of diseases. Following these presentations were questions regarding the changes with virulence of specific diseases over time. This was thought to be an important area for future research. The next section of the symposium addressed issues with mollusk culture. Dr. Seki's paper on abalone seed transplant emphasized the importance of resource enhancements of the algal communities present in the sea bottom ecosystem. Other participants talked about abalone endemic disease control in California, abolone changes in the feeding process and preference with growth, and the discovery of disease susceptibility in blacklip pearl oyster due to environmental changes such as gradual warming of the bay waters. Due to the symposium location in California several research topics of specific interest to the California aquaculture industry were also presented. A final paper on a bitter tasting amino acid in sea urchin ovaries stimulated a discussion on whether the occurrence of the acid was related to the natural progress of oogenesis or an unidentified factor in the natural diet of the sea urchin.

In conclusion, disease continues to be a major negative factor in the successful culture of crustaceans, mollusks and fish. Each year billions of dollars are lost world wide due to a host of diseases and parasites. Continuous exchange of information and technology at the international level must be done because human interactions lead to a broad dispersion of disease vectors around the globe. The UJNR continues to serve as an important contact venue

for both Japan and the United states on international disease issues and control. Several of the participants from both countries developed a dialogue
on how to work together in the future.

The Penaeid Shrimp Viral Pandemics due to IHHNV, WSSV, TSV and YHV: History in the Americas and Current Status

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Abstract

At least four virus caused pandemics have adversely affected the global penaeid shrimp farming industry since 1980. These viruses in the approximate order of their discovery are Infectious hypodermal and hematopoietic necrosis virus (IHHNV), Yellow Head Virus (YHV), Taura Syndrome Virus (TSV), and White Spot Syndrome Virus (WSSV). The socioeconomic impact of the diseases caused by these viruses have been so severe in some shrimp producing countries of Asia and the western hemisphere (Americas) that they were listed by the World Animal Health Organization (or 'Office International des Epizootics,' OIE) as posing a significant disease threat to cultured and wild crustaceans as a consequence of international trade or movement of infected crustaceans. IHHNV, TSV, and WSSV have had major impacts on cultured and wild shrimp in the Americas. Despite some unconfirmed reports, YHV has not been found in wild or cultured penaeid shrimp in the Americas. Nonetheless, YHV is considered to pose a significant threat to wild and cultured shrimp in the Americas because of the experimental demonstration of high susceptibility of American penaeids to the virus. Additional virus-caused diseases found in shrimp have been recognized recently. For example, Infectious Myonecrosis (IMN) has severely affected shrimp farms in northeast Brazil in 2003. This emerging disease was shown to have a viral etiology and the virus was named IMNV for the disease it causes. This paper reviews aspects of the biology and epizootiology of WSSV, TSV, IHHNV, YHV, and the emerging disease IMN.

Introduction

The shrimp farming industry of the Western Hemisphere (the Americas) produces approximately 25% of the world's farmed shrimp. With 91,000 metric tons (MT) of production for 2003, Brazil has become the region's leading producer. Brazil has thus replaced Ecuador which has not fully recovered from the WSSV pandemic of 1999, but still produced 81,400 MT in 2003 (I. Rocha, unpublished data, 'Presidente Assoccação Brasileria de Criadores de Camarão,' Recife, Brazil; Rosenberry 2003). In terms of significant production, only two farmed penaeid shrimp species have been significant in the Americas. Of these, *Litopenaeus vannamei* (the Pacific white shrimp; Perez Farafante and Kensley 1997) currently accounts for more than 90% of the total production. Other penaeid shrimp species have been cultured, and these make up the remaining percent of the total production. The second most important species is *L. stylirostris*, the Pacific blue shrimp, once accounted for nearly 20% of the hemisphere's production. However, the high susceptibility of the species to WSSV and to new strains of TSV resulted in the abandonment of the species in 1999-2000 by most of the industry (Zarin-Herzberg and Ascencio-Valle 2001; Erickson et al. 2002; OIE 2003). Also cultured in some locations are *L. setiferus* and *L. schmitti* (the Gulf of Mexico and Caribbean white shrimp, respectively) and *Farfantepenaeus aztecus*, *Fa. subtilis*,

and *Fa. californiensis* (the Gulf of Mexico, Caribbean, and Pacific brown shrimp, respectively) (Perez Farafante and Kensley 1997; Rosenberry 2001, 2003).

The most important disease of cultured penaeid shrimp, in terms of economic impact, in Asia, the Indo-Pacific, and the Americas have infectious agents as their cause (Flegel and Alday-Sanz 1998; Lightner 1999; OIE 2003). Among the infectious diseases of cultured shrimp, certain virus-caused diseases stand out as the most significant **(Tables 1 & 2).** Some of the most important diseases (and their etiological agents) were once limited in distribution to either the Western or Eastern Hemisphere (Lightner, 1996a; Flegel and Alday-Sanz 1998; OIE 2003). However, the international movement of live (for aquaculture) and dead (commodity shrimp for reprocessing and commerce) has led to the transfer and establishment of certain pathogens from one hemisphere to the other (Lightner 1996b; Durand et al., 2000; AQUIS, 2000). Frozen commodity shrimp have been implicated as the route by which WSSV was moved from Asia to the Americas, while TSV was moved in the opposite direction with infected live broodstock from Central America (Nunan et al. 1998; Tu et al. 1999; Yu and Song 2000; Durand et al. 2000).

Table 1. Viruses of concern to penaeid shrimp aquaculture in the Americas (as of January 2004; modified from Lightner 1996a and Lightner 1999).

Virus Type / Family	**Pathogen/Pathogen Group[1]**	
DNA VIRUSES		
dsDNA - *Nimaviridae*	* WSSV	- genus *Whispovirus*
dsDNA - *Baculoviridae*	** BP[2]	- an occluded enteric baculovirus
	** MBV[2]	- an occluded enteric baculovirus
	** BMN[2,3]	- a nonoccluded enteric baculovirus
ssDNA - *Parvoviridae*	** IHHNV	- a systemic parvovirus
	HPV	- enteric parvoviruses

* Listed by OIE (OIE 2003a, 2000b).

** Listed by OIE with "Other Significant Diseases" as of May 1999 (OIE 2000a, 2000b, 2001).

[1] For more information on these pathogens and the most appropriate diagnostic methods see: Lightner 1996 and OIE, 2003, Manual of Diagnostic Tests for Aquatic Animals.

[2] The most recent report on virus taxonomy from the International Committee on Taxonomy of Viruses (van Regenmortel et al. 2000) lists only MBV as a member of the baculovirus family. The omission of BP was almost certainly an oversight. Because BMN is nonoccluded, it was removed from the baculovirus family by the ICTV in 1995 (Murphy et al. 1995). Nonetheless, for the purpose of this list BMN, MBV, and BP will be listed a baculoviruses.

[3] BMN was de-listed in 2002 by OIE from its list of Crustacean Diseases. Nonetheless, it has been kept on the USMSFC list.

RNA VIRUSES		
ssRNA - *Dicistroviridae*	* TSV	- genus *Cripavirus*
ssRNA - *Roniviridae*	* YHV/GAV/LOV[34]	- genus *Okavirus*
dsRNA - *Totiviridae*	IMNV	- *Myonecvirus* (proposed new genus)

* Listed by OIE (OIE 2003a, 2000b).

** Listed by OIE with "Other Significant Diseases" as of May 1999 (OIE 2000a, 2000b, 2001).

Table 2. Estimated economic losses since the emergence of certain diseases in penaeid shrimp aquaculture.

Virus	Year of emergence to 2001	Product Loss (dollars)
WSSV - Asia	1992	$4-6 billion
WSSV-Americas	1999	>$ 1 billion
TSV	1991-92	$1-2 billion
YHV	1991	$ 0.1-0.5 billion
IHHNV*	1981	0.5-1.0 billion

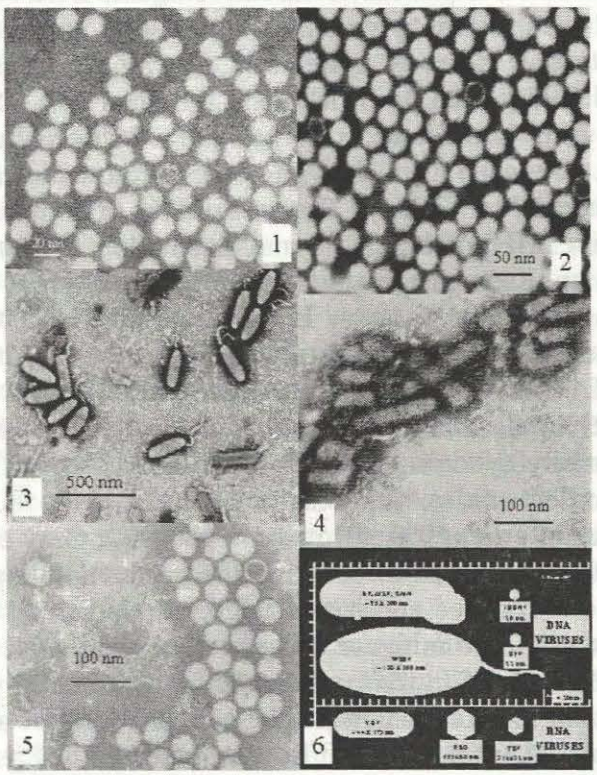
* Includes Gulf of California fishery losses for 1989-1994.

As a consequence of the rapid growth and development of the penaeid aquaculture industry, many of the most significant shrimp pathogens were moved from the regions where they initially appeared to new regions even before the "new" pathogen had been recognized, named, proven to cause the disease, and before reliable diagnostic methods were developed. The diseases caused bye to the shrimp viruses IHHNV, TSV and WSSV were all transferred with live shrimp stocks from country to country and from one continent to another well before their etiology was understood and diagnostic methods were available. The pandemics due to the penaeid viruses WSSV and TSV, and to a lesser extent to IHHNV and YHV, have cost the penaeid shrimp industry billions of dollars in lost crops, jobs, and export revenue **(Table 2)**. The social and economic impacts of the pandemics caused by these pathogens have been profound in countries in which shrimp farming constitutes a significant industry. This paper reviews the current status of the virus diseases due to WSSV, TSV, YHV, IHHNV and IMNV in the Americas in terms of their biology, history, distribution, production impacts, and methods for their management in shrimp aquaculture in the Americas. Details on diagnostic methods for these diseases (except IMN) and detection methods for their causative agents are available from several sources, with the most recent being Manual of Diagnostic Tests for Aquatic Animals (OIE 2003).

[4] The OIE (2003a, 2003b) lists YHV and includes GAV/LOV as a distinct strain of YHV.

The Major Viruses In The Americas

Although nearly 10 of the known shrimp viruses have been found in cultured penaeid shrimp in the Western Hemisphere **(Table 1)**, only four of these have caused panzootic disease with serious economic consequences in the Americas (Lightner 1996a, 1996b, 2003) during the past decade. These viruses, or perhaps more correctly groups of different strains of the same virus species, are IHHNV, TSV, WSSV, and IMNV **(Table 1; Figures 1-4 and 6)**. IMN (infectious myonecrosis; **Figure 5)** recently emerged in 2002-2003 in shrimp farms in the northeastern states of Brazil where it has caused serious mortality and production losses at affected farms (Lightner et al. 2004).



Figures 1-5. Transmission electron micrographs of purified (by ultracentrifugation in sucrose or cesium chloride gradients) preparations of penaeid shrimp viruses stained with 2% PTA. Scale bars are in nanometers as indicated on the figure plate. Fig. 1: IHHNV; Fig. 2: TSV; Fig. 3: WSSV; Fig. 4: YHV; and Fig. 5: IMNV.

Figure 6. Schematic of the principal viruses of penaeid shrimp grouped by nucleic acid type of the genome, and showing their relative sizes and their general profile. The spacing on the scale lines is 20 nm.

Infectious hypodermal and hematopoietic necrosis virus (IHHNV)

IHHNV is the smallest of the known penaeid shrimp viruses. The IHHN virion is a 22 nm diameter, nonenveloped icosahedron **(Table 1; Figures 1 and 6)**, with a density of 1.40 g/ml in CsCl. It genome is linear single-stranded DNA of 4.1 kb in length, and its capsid consists of four polypeptides with molecular weights of 74, 47, 39, and 37.5 kD. Because of these characteristics, IHHNV has been classified as a member of the *Parvoviridae* and a probable member genus *Brevidensovirus* (Bonami et al. 1990; Bonami and Lightner 1991; Mari et al. 1993; Nunan et al. 2000; Shike et al. 2000).

The disease IHHN, and later its causative agent, IHHNV, was first described as the cause of acute epizootics and mass mortalities (> 90%) in juvenile and subadult *L. stylirostris* farmed in super-intensive raceway systems in Hawaii (Brock et al. 1983; Lightner 1983, 1988; Lightner et al. 1983a, 1983b; Brock and Lightner 1990). Shortly after its discovery in *L. stylirostris*, the virus was found in *L. vannamei* being cultured at the same facility in Hawaii and these *L. vannamei* were shown to be asymptomatic carriers of the virus (Lightner et al, 1983b; Bell and Lightner 1984). Some members of populations of *L. stylirostris* and *L. vannamei* that survive IHHNV infections and/or epizootics, may carry the virus for life and pass the virus on to their progeny and other populations by vertical and horizontal transmission (Bell and Lightner 1984; Lightner 1996a; Morales-Covarrubias et al. 1999, Morales-Covarrubias and Chavez-Sanchez 1999; Motte et al. 2003). A few years after it was reported that *L. vannamei* could be infected with IHHNV and not present significant mortalities (Lightner et al, 1983b; Bell and Lightner 1984), IHHNV was shown to be the cause of 'runt deformity syndrome' (RDS) in *L. vannamei*. With RDS, irregular, reduced growth and cuticular deformities, rather than mortalities, were found to be the principal effect of infection (Kalagayan et al. 1991; Browdy et al. 1993; Bray et al. 1994; Brock and Main 1994; Lightner 1996a). Hence, the economic and production impacts of IHHNV infection in *L. vannamei* are due to reduced and irregular growth and small sized shrimp at harvest and not to elevated mortality. To mitigate this effect, several strategies have been used. With one strategy, selected lines of *L. stylirostris*, which were not only resistant to IHHN disease, but are also refractory to infection, were developed (Tang et al. 2000; Dhar et al. 2001). IHHNV-free lines of *L. vannamei* were also developed as SPF (specific pathogen-free) lines and these stocks were the first developed in the SPF stock development program (Pruder et al. 1995).

After its initial discovery in cultured shrimp in Hawaii in 1981, IHHNV was subsequently found to be widely distributed in cultured shrimp in the Americas and in wild shrimp collected along the Pacific coast. As of 2003, the only country in the Americas, which can claim to have IHHNV-free zones, is the United States. This was achieved with the development and use of SPF shrimp stocks (Pruder et al. 1995). The introduction of IHHNV into shrimp farms in northwestern Mexico and wild shrimp stocks in Mexico's Gulf of California during the late 1980's and early 1990's resulted not only in significant losses in farmed *L. stylirostris,* but also in a collapse in 1990 of the wild fishery for *L. stylirostris* in the northern Gulf of California (Lightner et al. 1992; Martinez-Cordova 1992; Lightner 1996b; Pantoja et al 1999; Morales-Covarrubias and Chavez-Sanchez 1999; Morales-Covarrubias et al. 1999). A decade later, the *L. stylirostris* fishery had recovered sufficiently to support commercial fishing, but the prevalence of IHHNV infection in adult *L. stylirostris* collected from the northern Gulf of California fishery remained high (80% to 100% females

and 60% in males) (Morales-Covarrubias et al. 1999; Morales-Covarrubias and Chavez-Sanchez 1999).

IHHNV has been found to be widely distributed in wild and cultured *Penaeus monodon* in east and SE Asia where it does not seem to cause production losses (Flegel 1997; Primavera and Quinito 2000; Tang et al. 2003). Molecular studies show considerable variation among Asian isolates of the virus (Tang et al. 2003), while little variation was found in Americas isolates (Tang and Lightner 2002). All isolates of IHHNV from the Americas are nearly identical with IHHNV from the Philippines, suggesting, along with other aspects of its history and epidemiology of IHHN in the Americas, its introduction from the Philippines, perhaps with live *P. monodon* that were imported as a candidate aquaculture species during the very early development of shrimp farming in the Americas (Lightner 1996b; Tang et al. 2003).

Taura Syndrome Virus (TSV)

TSV is a small, simple RNA virus. The TSV virion is a 32 nm diameter, nonenveloped icosahedron with a buoyant density of 1.338 g/ml **(Table 1; Figures 2 and 6).** The genome of TSV consists of a linear, positive-sense single-stranded RNA of 10,205 nucleotides, excluding the 3' poly-A tail, and it contains two large open reading frames (ORFs). ORF 1 contains the sequence motifs for nonstructural proteins, such as helicase, protease and RNA-dependent RNA polymerase. ORF 2 contains the sequences for TSV structural proteins, including the three major capsid proteins VP1, VP2 and VP3 (55, 40, and 24 kDa, respectively) (Bonami et al. 1997; Mari et al. 1998; Mari et al. 2002; Robles-Sikisaka et al. 2001). The virus replicates in the cytoplasm of host cells. Based on its characteristics, TSV has been assigned by the International Committee on Taxonomy of Viruses (ICTV) to the newly created genus *Cripavirus* in new family *Dicistroviridae* (in the superfamily of picoranviruses) (Mayo 2002a, 2000b).

The principal host for TSV is the Pacific white shrimp, *L. vannamei*, although other species can be infected and present disease (Aguirre and Valle 2000; Hasson et al. 1995, 1999; Lightner 1999; Overstreet et al. 1997; Robles-Sikisaka at al. 2001). Cumulative mortalities due to TSV epizootics have ranged from 40 to >90% in cultured populations of postlarval (PL), juvenile, and subadult *L. vannamei*. Survivors of TSV infections may carry the virus for life (Brock et al. 1995, 1997; Hasson et al. 1999; Lightner 1996a, 1996b; Lotz 1997). TSV has been demonstrated to remain infectious in the feces of sea gulls that have ingested infected shrimp carcasses, which may implicate birds as being an important route of transmission of the virus within affected farms or farming regions (Garza et al. 1997; Lightner 1999). TSV can also infect other Western Hemisphere penaeid species (i.e. *L. stylirostris*, *L. setiferus*, and *L. schmitti*), sometimes resulting in disease and mortalities in PL or yearly juvenile stages, but also in asymptomatic persistent infections (Brock et al. 1997; Overstreet et al. 1997). Other Western Hemisphere penaeids (*Farfantepenaeus aztecus* and *Fa. duorarum*) and Eastern Hemisphere penaeids (*Fenneropenaeus chinensis*, *P. monodon*, and *Marsupenaeus japonicus*) have been experimentally infected with TSV (Brock et al. 1997; Overstreet et al. 1997).

TSV emerged from an unknown source in Ecuador in 1991. The disease was recognized as a major new disease of farmed *L. vannamei* by early 1992 and it was named Taura Syndrome (Jimenez 1992; Lightner et al. 1995). The viral etiology of TS was

confirmed in 1994 and the virus was named Taura syndrome virus (TSV) (Hasson et al. 1995). In the interest of supporting litigation brought by a group of Ecuadorian shrimp farmers against several companies that had been implicated as the cause of a toxicity syndrome they called 'Taura Syndrome' (Intriago et al. 1997), Jimenez et al. (2000) reported on the epizootiology of the disease in Ecuador assigned to TSV the synonym infectious cuticular epithelial necrosis virus (ICENV).

By 1994, when the viral etiology of TS had been established, the virus had been moved with live shrimp transfers to many of the shrimp growing countries of the Americas (Brock et al. 1995; Hasson et al. 1995, 1999; Bonami et al. 1997; Lightner 1996a, 1996b). While wild postlarvae with TSV infections were reported as being found near shrimp farms with ongoing TSV epizootics (Lightner et al. 1995), TSV infections in wild shrimp have not been further documented, suggesting that TSV does not have a discernable impact on wild populations of shrimp (Brock 1997). By 1998, TSV reached Asia with infected stocks of *L. vannamei*, introduced for aquaculture purposes (Tu et al. 1999; Yu and Song 2000). Physicochemical and more recent molecular studies of TSV suggest that a single strain of the virus was present in the initial TSV pandemic, but that new strains are emerging which differ in host range and virulence (Yu and Song 2000; Zarin-Herzberg and Ascencio-Valle 2001; Erickson et al. 2002).

White Spot Syndrome

WSSV has a wide host range among decapod crustaceans (Lo et al. 1996; Flegel 1997; Flegel and Alday-Sanz 1998), and is potentially lethal to most of the commercially cultivated penaeid shrimp species (OIE 2003). The causative agent of WSD is white spot syndrome virus (WSSV) or white spot virus (WSV), is a very large double-stranded DNA (dsDNA) virus recently assigned by the ICTV to its own new genus, *Whispovirus*, and family, *Nimaviridae* (Table 1) (Mayo 2002a, 2002b). Virions are large (80-120 x 250-380 nm), rod-shaped to elliptical, and with a trilaminar envelope (Wang, et al. 1995; Durand et al. 1997; Inouye et al. 1994, 1996; Kanchanaphum et al. 1998; van Hulten, et al. 2001). Negatively stained virions purified from shrimp hemolymph show unique, tail-like appendages (Figures 3 and 6) (Wang et al. 1995). The virions are generated in hypertrophied nuclei of infected cells without the production of occlusion bodies. In initial reports, WSSV was described as a non-occluded baculovirus, but WSSV DNA sequence analysis has shown that it is not related to the baculoviruses (van Hulten et al. 2001; Yang et al. 2001). The size of the WSSV genome has been differently reported for different isolates: 305,107 bp (GenBank Accession No. AF332093), 292,967 bp (GenBank Accession No. AF369029) and 307,287 bp (GenBank Accession No. AF440570) for viruses isolated from the People's Republic of China, Thailand and Taipei China, respectively. The sequences of these three isolates are almost identical, with the size differences being due mostly to several small insertions and one large (~12 kBp) deletion. In accordance with a genome size of ~300 kb, a total of 531 putative open reading frames (ORFs) were identified by sequence analysis, among which 181 ORFs are likely to encode functional proteins. Thirty-six of these 181 ORFs have been identified by screening and sequencing a WSSV cDNA library or else have already been reported to encode functional proteins many of which show little homology to proteins from other viruses (OIE 2003).

White spot disease (WSD caused by WSSV) emerged in east Asia in 1992-93 and it was quickly dispersed with infected seed and broodstock across the Asian continent to SE Asia and India where it caused a major pandemic, and continues to cause significant losses in some regions. WSD outbreaks were first reported from farmed Ma. *japonicus* in Japan in 1993 (Inouye et al. 1994, 1996; Nakano et al. 1994) and the causative agent was named penaeid rod-shaped DNA virus (PRDV) or rod-shaped nuclear virus of *Ma. japonicus* (RV-PJ). Later, outbreaks of viral disease with similar gross signs caused by similar rod-shaped viruses were reported from elsewhere in Asia and other names were applied: hypodermal and hematopoietic necrosis baculovirus (HHNBV) in the People's Republic of China (Huang et al. 1995a, 1995b); white spot baculovirus (WSBV) and PmNOBIII in Taipei China (Chou et al. 1995, Lo et al. 1996); and systemic ectodermal and mesodermal baculovirus (SEMBV) or PmNOBII in Thailand (Wongteerasupaya et al. 1995). The virus from the People's Republic of China has also been called Chinese baculovirus (CBV) (Lu et al. 1997). Shrimp exhibiting the gross signs and histopathology of WSD have also been reported from Korea (Kim et al. 1998), India (Karunasagar et al. 1998), the Philippines, and the USA (Lightner 1996; Durand et al. 2000). WSSV has even reached shrimp farms in southeastern Europe (1997) and the Middle East (1999) via live shrimp movements, and Australia and Spain with introductions of frozen infected shrimp that were used as fresh food for broodstock (OIE 2003; Lightner unpublished data).

During 1999, WSD also had a severe impact on the shrimp industries of both Central and South America (Durand et al. 2000; Vidal et al. 2001; Lightner 2003). Despite the absence of evidence of live shrimp introductions from Asia to the Americas, WSSV was diagnosed at several sites in 1995-1997 in captive wild shrimp or crayfish and in cultured domesticated shrimp stocks in the eastern and southeastern U.S. (Nunan et al. 1998; Durand et al. 2000; Lightner et al. 2001). Early in 1999, WSSV was diagnosed as the cause of serious epizootics in Central American shrimp farms. By mid to late 1999, WSSV was causing major losses in Ecuador (then among the world's top producers of farmed shrimp), and by 2000-01, export of shrimp from Ecuador was down nearly 70% from pre-WSSV levels (Rosenberry 2001; Lightner 2003). Although the documentation is sketchy, WSSV has been found in wild shrimp stocks in the Americas (Nunan et al. 2001). In the US, the virus was successfully eradicated from shrimp farms and it has not been reported from farmed shrimp stocks since 1997. However, its sporadic detection in wild shrimp stocks (Gulf of Mexico and SE Atlantic states) (Thomas McIlwain, U.S. DOC, NOAA, National Marine Fisheries Service, St. Petersburg, FL, unpublished data) suggests that it has become established in wild penaeid shrimp stocks in SE US coastal waters or that it continues to be introduced perhaps, with wastes (peeled shells, etc.) from value-added reprocessing of imported shrimp in coastal packing plants. It has been proposed that the introductions of WSSV to the Americas were the result of importation of frozen shrimp products from WSSV-affected areas of Asia and the value-added reprocessing of those frozen shrimp for the US market in coastal processing plants (Nunan et al. 1998; Durand et al. 2000; Lightner et al. 2001; Lightner 2003). WSSV also reached Spain and Australia in 2000-2001. In both cases successful containment and eradication was reported, and for both events the importation and use of infected frozen shrimp as a fresh feed for broodstock was implicated as the route of introduction. Regardless of where they were obtained, isolates of WSSV have shown little genetic or biological variation, suggesting that the virus emerged and was spread from a single source (OIE 2003).

Yellow Head Disease

Yellow head disease (YHD) was first described in 1991 as an epizootic in Thai shrimp farms (Limuswan 1991), and subsequent outbreaks have been reported from other shrimp farming countries in Asia. A closely related strain of YHV, named Gill-Associated virus (GAV), has been reported from Australian shrimp farms (Walker et al. 2001). Laboratory trials have shown that YHV can cause high mortality in representative cultured and wild penaeid species from the Americas (Lu et al. 1994, 1997; Lightner 1999; Pantoja & Lightner 2003). When it occurs in farms rearing *P. monodon*, YHD is characterized by high and rapid mortality that is sometimes accompanied by the gross signs of yellowing of the cephalothorax and general bleaching of body color from which the disease got its name. In laboratory studies, American penaeids challenged with YHV did not develop yellow heads or signs of marked discoloration (Lightner and Redman 1998). YHV is potentially lethal to most of the commercially cultivated penaeid shrimp species (OIE 2003).

The causative agent of YHD is YHV, GAV and other closely related strains of the same virus (Table 1) (OIE 2003). Transmission electron microscopy (TEM) of YHV-infected tissues shows enveloped bacilliform virions. They range from approximately 150 nm to 200 nm in length and from 40 nm to 50 nm in diameter and are located within vesicles in the cytoplasm of infected cells and in intercellular spaces. The virions arise from longer, filamentous nucleocapsids (approximately 15 nm x 130-800 nm), which accumulate in the cytoplasm and obtain an envelope by budding at the endoplasmic reticulum into intracellular vesicles. Negatively stained YHV virions show regular arrays of short spikes on the viral envelope (Figures 4 and 6) (Boonyaratpalin et al. 1993; Chantanachookin et al. 1993; Lightner 1996a).

YHV was originally described mistakenly as a granulosis-like virus (Boonyaratpalin et al. 1993; Chantanachookin et al. 1993), but it was later found to be a single-stranded, positive sense RNA (ssRNA) virus (Tang and Lightner 1999) related to nidoviruses in the Coronaviridae and Arteriviridae (Sittidilokratna et al. 2002). GAV, the Australian strain of YHV has been recognized as the type species for the new virus genus *Okavirus* in the new family *Roniviridae* (Mayo 2002a, 2002b; OIE 2003).

Although YHD was first described as an epizootic from Thai shrimp farms (Limsuwan 1991), subsequent outbreaks of YHD have been reported from cultivated shrimp in many locations in Asia (OIE 2003). YHV has also been reported in frozen imported commodity shrimp in the United States (Nunan et al. 1998; Durand et al. 2000), and it has been incorrectly reported in farmed shrimp from the Americas based on the presentation of severe necrosis of the lymphoid organ, a lesion once thought to be pathognomonic for YHD (Lightner 1996a; Lightner et al. 1998; Lightner and Redman 1998) . However, the diagnosis of YHV infection in these cases was not confirmed with a second diagnostic method until after the errant reports were published. More recent work has shown that the presumptive histological diagnoses were due to severe infections with white spot virus, which can cause histopathology in the lymphoid organ which mimics that occurring in severe acute YHD (Pantoja and Lightner 2003). Because of the risk of introduction of YHV into the Western Hemisphere with frozen commodity shrimp still remains (Nunan et al. 1998, Durand et al. 2000), and because the possibility that concurrent WSSV/YHV infections may occur, in those severe WSSV cases in which YHV infection may also be suspected, the samples

should be further analyzed by another method (i.e. RT-PCR or ISH with a YHV specific probe) to confirm or rule out the presence of YHV. Hence, despite some early reports of YHV in the Americas, it has not been found infecting farmed shrimp in the Americas.

Infectious Myonecrosis (IMN)

Infectious myonecrosis (IMN) is a recently identified disease in cultured *L. vannamei* in northeast Brazil (Table 1). IMN causes significant disease and mortalities in juvenile and subadult pond-reared stocks of *L. vannamei*. In 2003, IMN is reported to have been responsible for millions of dollars in losses in northeast Brazil (unpublished data, ABCC, Brazil). Outbreaks of the disease seemed to be associated with certain types of environment and physical stresses (i.e. extremes in salinity and temperature, collection by cast net, etc.), and possibly with the use of low quality feeds. IMN presents as a disease in *L. vannamei* with an acute onset of gross signs and elevated mortalities, but it progresses with a more chronic course accompanied by persistent low level mortalities. To date, IMN appears to be limited to northeast Brazil, but shrimp with similar gross signs have been also reported from other countries where *L. vannamei* are cultured. Affected shrimp present extensive white necrotic areas in the striated muscle, especially of the distal abdominal segments and tail fan. These may become necrotic and reddened in some individual shrimp. By histopathology, shrimp with acute phase disease present lesions with coagulative muscle necrosis, often with edema. In shrimp recovering from acute disease or those in the more chronic phase of the disease, the myonecrosis appears to progress from coagulative to liquefactive necrosis. This progression of myonecrosis is accompanied with hemocytic infiltration and fibrosis. Significant lymphoid organ spheroid formation is typically present, and ectopic lymphoid organ spheroids are often found in the hemocoel and loose connective tissues, especially in the heart lumen and adjacent to antennal gland tubules. In some histological preparations, perinuclear pale basophilic to darkly basophilic inclusion bodies are evident in muscle cells, connective tissue cells, hemocytes, and in cells that comprise lymphoid organ spheroids (Lightner, unpublished data).

The infectious nature of the disease has been demonstrated by transmission of the disease into SPF indicator shrimp by injection and per os challenge studies using cell-free filtrates prepared from diseased shrimp or chopped diseased shrimp carcasses, respectively. A 40 nm diameter spherical virus has been isolated from naturally infected shrimp with the disease (Figure 5). The virus has been partially characterized and portions of its nucleic acid (RNA) genome have been cloned and sequenced. Molecular probes and RT-PCR methods for diagnosis of the disease and detection of IMNV have been developed and will be reported elsewhere (Lightner et al. 2004).

Disease Management

Until the WSSV pandemic, the penaeid shrimp farming industry in Asia and the Americas remained largely dependent on wild shrimp for stocking its farms. This was accomplished by the practice of collection and use of wild seed (postlarvae) for stocking of its farms directly or by the use of captive wild broodstock for the production of seed stock in hatcheries. This dependence has fostered the intensification and spread of the viral diseases in shrimp aquaculture and in wild populations. The shrimp farming industry as a whole has

recognized this fact and it has begun to change its farming practices in order to continue to develop, if not survive. While many of the shrimp stocks currently used to stock farms are produced from captive wild broodstock, only those that test negative for WSSV in Asia and WSSV and TSV in the Americas are used to stock biosecure farms. Biosecure production systems (that are designed to exclude potentially infected wild shrimp seed) stocked with shrimp stocks known to be free of the major shrimp pathogens have become a common practice in many shrimp growing regions. A further sign of a maturing industry is its movement towards the expanded development and use of specific pathogen-free (SPF) domesticated shrimp stocks of the most important shrimp species (Pruder et al. 1995; Moss et al. 2002; Lightner 2003; Lee and O'Bryen 2003). The techniques have advanced shrimp farming and made the industry far more sustainable than it was before the emergence of the virus caused diseases discussed in the present paper.

Acknowledgments

Grant support for much of the work cited in this review was provided by the United States Marine Shrimp Farming Consortium CSREES, under Grant No. 99-38808-7431, and by a series of special grants from the National Fisheries Institute.

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Occurrence and Prevention of White Spot Syndrome (WSSV) in Malaysia

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Key Word: WSSV, viral disease, PCR, Malaysia, disinfectants

Abstract

Southeast Asia is a significant area for world shrimp culture. However, in recent years, the production of cultured shrimp has markedly decreased as a result of serious viral disease outbreaks. Especially, the increased severity of widespread White Spot Syndrome Virus (WSSV) infection is the most serious threat to stable aquacultural production. In the case of Malaysia, outbreak of this disease was almost the same as Thailand situation that was WSSV disease occurring as serious problem from 1996. A lot of farms have given up shrimp culture due to heavy loss incurred by WSSV until 1997. Field research was performed through 1998 and 1999 on the occurrences of WSSV at farms in Malaysia. Dark-field microscope observation and Japanese PCR methods for PRDV detection were used for this research, because both methods were confirmed to use for detection of Malaysian strain of WSSV. From this research, WSSV outbreaks were found in Penang, Keda and Sarawak State, and information of the occurrence of viral disease was obtained. Almost all prawns in the ponds were dead by this disease. These dead prawns were showing many clear White spots on their carapace. Pathogenic viruses were confirmed by PCR from the samples WSSV. In this etiological study, as for the infection routes, it could be supposed two ways. One was that the prawn fry had been infected with this virus, and another was from supplied seawater containing the virus particles. As one of the preventive countermeasures against viral diseases, inactivation methods for WSSV were studied. This viral inactivation was tested with formalin and halogenous disinfectants and sodium hypochlorite. These chemicals were mixed with the virus and injected to the healthy prawns. As a result of these experiments, no mortality was shown using0.25% effective chloride in sodium hypochlorite and 0.5 ppm formalin. From these results, sodium hypochlorite of halogenouse disinfectants showed the effective inactivation even under lower concentration. It was suggested that these disinfectants were extremely useful for the WSSV inactivation.

Introduction

Southeast Asia is an area of great significance in world shrimp culture. However, in recent years, the production of cultured shrimp has markedly decreased as the result of serious viral diseases. The increased severity of widespread White Spot Syndrome Virus (WSSV) infection is the most serious threat to stable aquacultural production. Features of this WSSV were that the diseased prawns were often showing obviously White spots on their carapace and that the high mortality was occurred from 80% to 100% in only few days after infection (Nakano *et. al.* 1994, Chou *et. al.* 1995).

The first occurrence of WSSV was in Taiwan in 1992 (Chou *et. al.* 1995). In Japan it first appeared in 1993, a new acute viral disease occurred at the kuruma prawn farm (Nakano *et. al.* 1994). These prawns were imported from China as fry. This disease was called Penaeid Acute Viremia (PAV) in Japan and the pathogenic virus was named Penaeid Rod-shaped DNA virus (PRDV) from their particle shape (Inouye *et. al.*1996). WSSV was

also reported in 1993 in Korea (Park *et. al.* 1998) and the disease spread into Thailand in 1994 (Wongteerasupaya *et. al.* 1995). Furthermore, this disease occurred in India in 1994 and 1995 (Karunasagar *et. al.* 1997), and lastly, it was recognized in the Philippines in 1999, which was far away from other east and Southeast Asian countries (Magbanua *et.al.* 2000). Since WSSV occurred at first in 1992, this disease has spread among most Asian countries. Recently, WSSV has spread widely in the world, not only in Asian countries, but also in the USA (Lightner 1996), Central America and South America (O.I.E. 2003). In the case of Malaysia, WSSV was reported in 1994 at almost the same period that Thailand reported the disease (Wang *et. al.* 1999). It was estimated that this disease reached Malaysia through importation of the post larvae from Thailand. WSSV spread rapidly in the peninsular Malaysia, and the disease caused enormous economic losses. The outbreaks of this disease became the most serious problem facing prawn culture farms in Malaysia since 1996. In such conditions, the international collaborative project "Development of Technology for the Diagnosis and Prevention of Shrimp Viral Diseases" was planned by the Japan International Research Center for Agricultural Sciences (JIRCAS). This project was carried out in Malaysia by the Fisheries Research Institute (FRI), Department of Fisheries, Ministry of Agriculture, Malaysia. This project investigated the occurrence of prawn diseases in Malaysian farms and viral inactivation techniques by using disinfectants for disease control.

Application for the Existing Diagnostic Method for WSSV

Many diagnostic methods for WSSV were already been reported. In these methods, the dark-field microscopic observation method which were reported by Momoyama *et. al.* (1995) is a quite simple and easy method for detecting this disease. The advantage of this method is that diagnosis could be performed in the ordinary laboratory without expensive and specific machines, and it needed only a biological microscope with wet-type dark-field capacitor. But, this method was adapted only for the case of Kuruma prawn, so that it s needs to be tested for WSSV of black tiger prawn. The PCR method is the most popular diagnosis for detection of WSSV. These methods provide an accurate diagnosis for virus specific genomes. However, new strains sometimes cannot be detected by this method because of minor distinguishable factors between even local strains. For these reasons, it was necessary to confirm whether, local strains of WSSV in Malaysia could be detected by these both diagnostic methods of PAV used in Japan.

(a) Dark-Field Microscopic Observation

This method was tested using hemolymph and stomach samples, collected from prawns artificially infected prawns with WSSV. The stomach samples were provided with raw and 10% formalin fixed tissues. The observation method was followed Momoyama *et. al.* (1995). A lot of small and shiny particulates were observed in the hemolymph samples of diseased prawns. These particulates may be one or more agglutinated particles of WSSV. However, the stomach did not show the same observable features because the epidermal layer could not be peeled easily from the cuticula. From these results, hemolymph was suitable for simple diagnosis of WSSV using dark-field microscopic observation. On the

other hand, this method might led to incorrect diagnostic results because it is difficult to distinguish between the particles of WSSV and the small dust. Therefore, we will need to confirm this viral disease using other accurate diagnostic methods as a counter-check.

(b) PCR Diagnosis Method

PAV detection method of Kimura *et. al.* (1996) was used in this study. This PCR diagnosis method is used in Japan and was developed jointly at Fisheries Research Agency, National Research Institute of Aquaculture (NRIA) and Kumamoto Prefectural Fishery Research Center. Sample DNA was extracted from the muscle of diseased prawns infected with WSSV in Penang Island in August 1998. These DNA were prepared in ten-fold serial dilutions until 10^{-9} and each of the diluted fluids was applied to PCR. Two pairs of PCR (P1-P2, P3-P4) were used in this test, because this was a nested PCR method requiring two-step PCR procedures. The results of PCR, analysis showed that the band was found at the same location in agar gel as the PRDV (about 560 base pair). The detection limit of WSSV was 5-plex (10^{-5}) dilution of the virus sample at the nested step although virus was detected under 3-plex (10^{-3}) diluted samples at the single step alone. These results were showed that both PRDV and this local strain of WSSV were quite similar; at least the regions of primer sequences were almost same. It was very clear that the PAV detection method was useful for screening of the Malaysian local strain of WSSV.

Field Research on WSSV Occurrence in Malaysia

The prawns were mainly produced in the area located on the western coast and southern part of the Peninsular Malaysia. The places on the western coast were Penang, Perak, and Kedah State centering Penang City, and Selangor State centering on Kuala Lumpur City. The southern area was Johor State centered Johor Bahr City. On the eastern coast, prawn production was not as successful. One of the causes must be that prawn ponds were damaged by heavy rain and strong wind in the monsoon seasons. Another cause may be economical. Prawn consumption in this area was lower than west coast with big cities. However, the prawn industry has begun to develop rapidly in the Saba and Sarawaku State on Borneo Island recently. There was a large space for culturing prawns in vast mangroves area there. WSSV became serious problem in the prawn farms starting in 1996, mirroring the same situation of Thailand. Damages were seriously in the Keda State, which was showing a lot of neglected ponds all over. In contrast, YHV disease was not reported in Malaysia although it was a serious problem in Thailand.

Field research occurred in 1998 and 1999 on the occurrences of WSSV at Malaysian fish farm. The diagnoses for WSSV were used with dark-field microscopical observation and PCR, but confirmation of the virus was carried out using PCR of PAV method. Field research sites and periods were shown in **Table 1**.

Area		Main period for investigation	
State	***place***	***1998***	***1999***
Northern part of east coast in Peninsula Malaysia			
Keda State	Sungai Patani	Feb. Jun Aug.	Jan. March*[1]
Penang State	Penang Island	Feb. Jun Aug. *[1]	Jan.
	Butterworth		Jan.
Perak State	Nibong Tebal	Jun Aug.	Jan.
	Taiping		Jan.
Southern Part of Peninsula Malaysia			
Johor State	Gran patha		Aug.*[2]
	Johor Bahru		Jan.
	Batu Pahat		Jan.
Borneo Island			
Sarawak	Kuching		May*[1,2]

*1: WSSV occurrence was confirmed in the field investigation.
*2: Delivery samples

Table 1. Field research of the prawn culture farms in Malaysia

In 1998, field research was carried out in February, June and August, and research took place at Penang Island, Nibong Tebal, and Sungai Patani, in Penang, Perak, and Kedah State, respectively (**Fig.1**). In January of 1999, investigations also took place at Taiping in Perak State, Bukit Tambun in Penang State, and Johor Bahru and Batu Pahat in Johor State. Samples from Kuching of Sarawak State were checked on March and May 1999. This Sarawak State was well known to be free of White Spot Syndrome Virus (WSSV) before the study. A lot of farms have given up shrimp culture due to heavy loss incurred by WSSV until 1997. Many shrimp farmers changed their ponds into fishing ponds. In this field research, WSSV was detected at prawn farms of Penang Island in 1998 and killed almost all prawn ponds (**Fig.2**). The dead prawns showed many clear White spots on their carapace (**Fig. 3**). The prawn samples for PCR were collected from Keda, Pera, and Penang State in February, June, and August 1998 and January 1999.Samples from Sungai Patani of Keda state at March and from Sarawak at May in 1999 underwent a PCR analysis. From the result of PCR test showed no virus was detected in the samples collected at Keda, Pera, Penang State in January, 1999. WSSV positive results were found in Penang in August, 1998, Keda in March 1999 and at Sarawak, where WSSV were outbreaks had already been reported. In this field research, viral disease confirmed by WSSV was confirmed for Penang, Sungai Patani and Kuching on August 1998, March and May 1999, respectively. The other research places did not encounter WSSV disease. In this etiological study, it was clear that WSSV came into these farms by two routes. One route was that pathogenic viruses originated from shrimp fry since the disease occurred one month after fry introduction into the pond. WSSV also came via supplied seawater from the ocean because the disease broke out at the rainy season when the seawater was stirred. The culture environments culture also affected the disease outbreak. The high density of the prawns associated with intensive culture system contributed to the spread of WSSV. The quite low water exchange rate also affected the disease outbreak. The low salinity, which was occasionally almost fresh water in rainy season, and affected the disease outbreak.

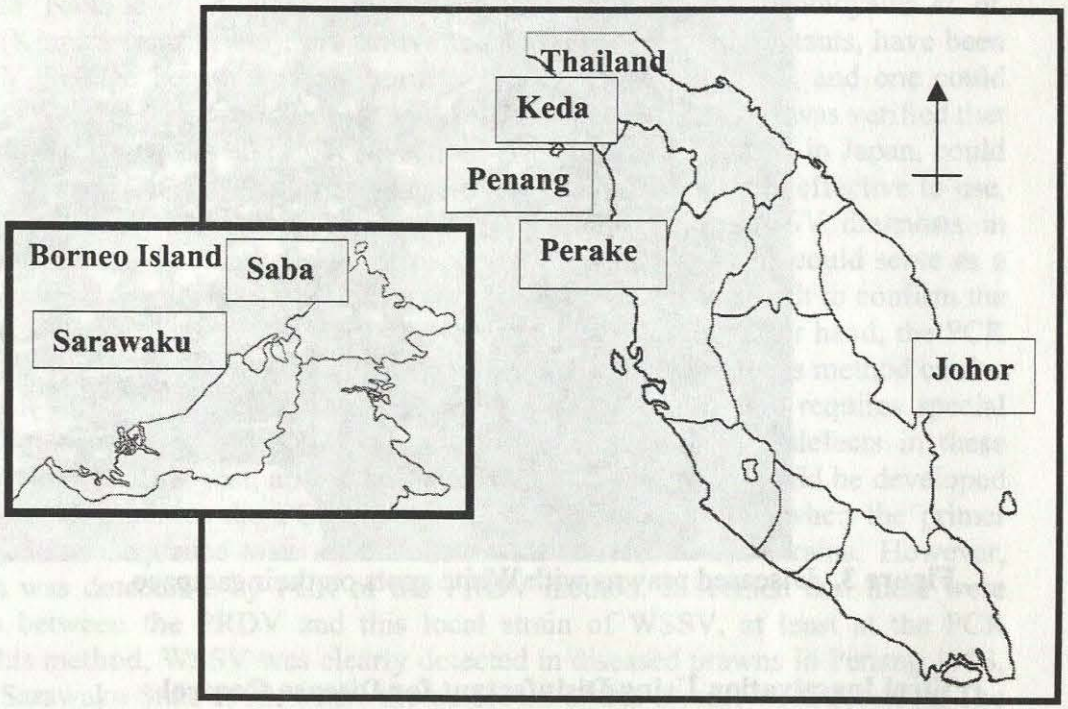

Figure 1. Map of Malaysia

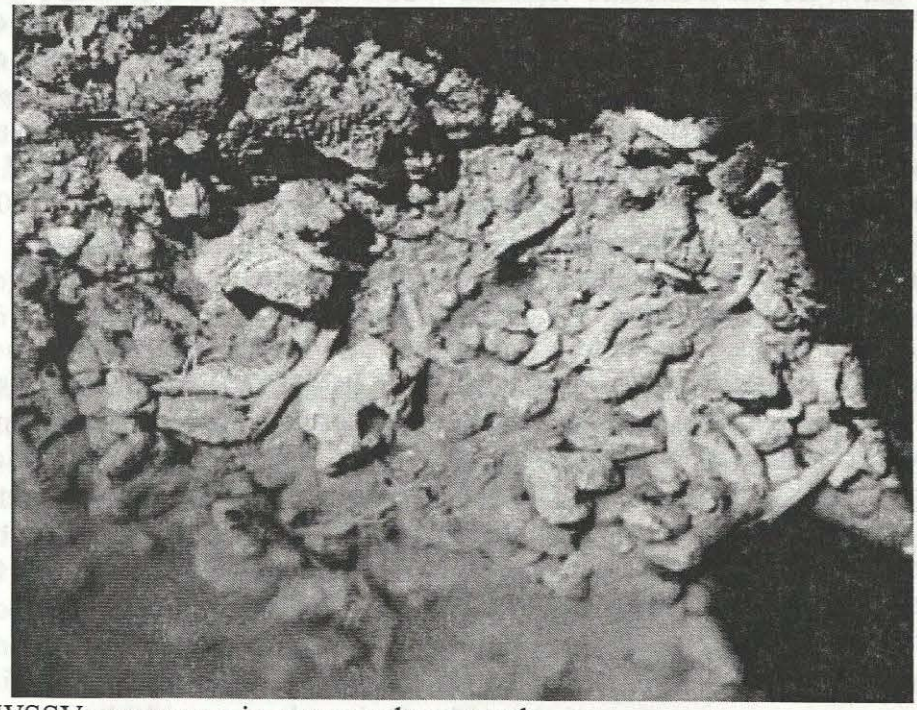

Figure 2. WSSV occurrence in prawn culture pond.

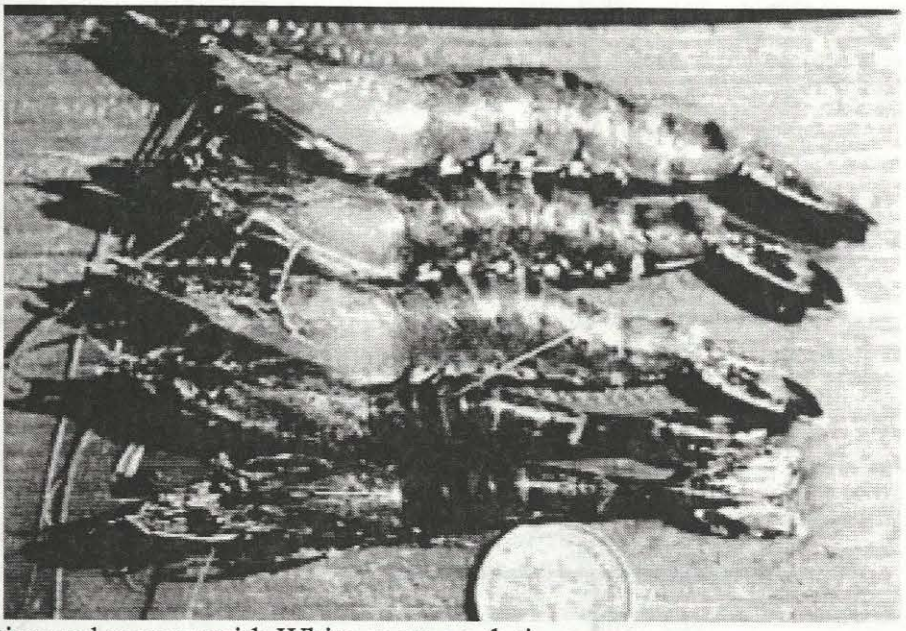

Figure 3. Diseased prawns with White spots on their carapace.

Viral Inactivation Using Disinfectant for Disease Control

Disinfectants are mainly used as a preventive measure to protect against the introduction of pathogens. The methods of inactivation for viral pathogens were developed using general disinfectants in this study. Viral inactivation was tested using chemicals, such as formalin and sodium hypochlorite of halogenous disinfectants. These chemicals were mixed with the virus at 25°C. After the reaction, the resultant products were injected intramuscularly, and the mortality was monitored for 2 weeks after the injection. Formalin, which was used to exterminate fish parasites, was prepared concentrations of 0 to 1% (V/V). Each formalin solution was mixed with virus fluid and reacted together for 10 minutes. After that, the solution was diluted to stop the reaction. Sodium hypochlorite that is used in halogenous disinfectants, was added into the diluted virus fluid and reacted together for 10 minutes. The final concentrations of effective chloride were from 0 to 5.0ppm. These reactions were stopped by sodium thiosulfate. The reactants were then used for artificial infections. No mortality was shown when over 0.25% (V/V) formalin was used. Thus, it became clear that this virus was inactivated by more than a 0.25% formalin concentration. On the other hand no mortality was shown over 0.5 ppm for sodium hypoclorite. Thus, this virus was inactivated by only 0.5ppm of effective chloride concentration in sodium hypochlorite. From these results, sodium hypochlorite of halogenous disinfectants induced an effective inactivation at even lower concentrations. So, it was suggested that this disinfectant was extremely useful for the inactivation of WSSV.

Discussion

The main Malaysian prawn species was the black tiger prawn. There was intensive culture and few water exchanges in the rearing of this species. This culture style was similar between Thailand and Malaysia. These bad environmental conditions caused the prawn

diseases. In Japan, PAV was occurred in the cultured kuruma prawns in 1993, and it became a serious problem (Nakano *et. al.* 1993). The PRDV diagnostic method (Momoyama *et. al.* 1995) and PCR (Kimura *et. al.* 1996), preventive techniques by the disinfectants, have been developed WSSV damage became serious starting in 1996 for Malaysia., and one could hypothesize that it had the same pathogenic origin as PRDV. In this study, it was verified that the methods of simple diagnosis and PCR detection, which were developed in Japan, could be adapted to Malaysia. It became clear that these diagnostic methods were effective to use, and these simple or conformed diagnosis could be carried out for WSSV diagnosis in Malaysia as well in Japan. The dark-field microscopic observation method could serve as a simple WSSV diagnosis for heavily infected shrimp. However, it was difficult to confirm the virus with this method and the detection sensitivity was lower. On the other hand, the PCR method has been proven as a useful WSSV diagnostic method. However, this method carries a high cost, and it takes a long time to get the diagnostic result. It also requires special techniques and equipments to run. Therefore, there are advantages and defects in these various methods. Rapidly, low cost, and accurate diagnostic techniques should be developed in the future. Also, Sometimes the PCR method t could not be reacted when the primer setting of the genome sequence was mismatched with different virus strains. However, Malaysian strain was detectable by PCR of the PRDV method. It seemed that there were fewer variations between the PRDV and this local strain of WSSV, at least at the PCR regions. Using this method, WSSV was clearly detected in diseased prawns in Penang 1998, Keda 1999, and Sarawaku State 1999, where the outbreaks of this disease were occurred. The virus could not be detected in other areas. Thus not all prawn farms were necessarily polluted by WSSV, or that all surviving prawns became virus carriers. Almost all prawn farmers gave up managing ponds due to the high WSSV damage. Therefore, the surviving farmers are either virus free or good managers.

In this etiological study, there were two assumed infection routes. One infection route was that the prawn fry had been virus carriers, and another theory is that the virus came from added seawater that contained the virus particles. These results are important for preventing a WSSV epidemic. The disease occurrence must be controlled by screening of the shrimp fry and by disinfecting supplied seawater. Recently, the inspections of prawn fry using PCR have been carried out actively at the fisheries institute of government and by private companies. The occurrence of WSSV has decreased for this reason. However, almost all prawn fry were not tested because of the high cost. Thus, the disease still continues to occur.

Nakano *et. al.* (1998) described that halogenous disinfectants were quite useful for PRDV inactivation. From these results of WSSV, sodium chloride of halogenous disinfectants also induced an effective inactivation for this virus even at lower concentrations. It was suggested that these disinfectants were extremely useful for the inactivation of WSSV. Some farmer began to use the chlorine to sterilize the supplied water in the reserve ponds. But, usage of these disinfectants should be limited to sterilizing of culturing tools and gear, or pond water at lower concentration. This chemical is not only toxic for the virus, but also for the prawns. This study should be continued, because the WSSV disinfections prevented pathogen intrusion at aquaculture farms and thus has contributed to sustainable aquaculture in Southeast Asia.

Acknowledgments

I appreciate Dato' Hashim Bin Ahmad, Director General of Department of Fisheries Malaysia (DOF), for approving this collaborative project. I also appreciate Mr. Ismail Awang Kechik, Director of Fisheries Research Institute (FRI), for arrangement of my research. I thank Mr. Veloo Palanisamy, section head of fish health in FRI, Mr. Chuah Toh Thye, the other staffs of fish health section in FRI, and also staffs of JIRCAS Penang Office.

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Disease Prevention Strategies for Penaeid Shrimp Culture

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Abstract

Penaeid shrimp aquaculture expanded significantly over the past two decades. However, shrimp farmers have suffered significant economic losses because of viral diseases. Researchers from the U.S. Marine Shrimp Farming Program (USMSFP) have developed novel approaches to mitigate the devastating impact of shrimp viruses, including the use of specific pathogen free (SPF) and specific pathogen resistant (SPR) shrimp, as well as the establishment of biosecure production systems that rely on pathogen exclusion. These approaches have evolved over the past decade in response to changing disease problems faced by U.S. shrimp farmers. In the late 1980's and early 1990's, U.S. farmers observed Runt Deformity Syndrome (RDS), an economically significant and frequent disease problem of cultured Pacific white shrimp, *Litopenaeus vannamei*. RDS is characterized by reduced growth rates and cuticular deformities and is caused by Infectious hypodermal and hematopoietic necrosis virus (IHHNV). The increasing incidence of RDS on commercial farms catalyzed USMSFP researchers to develop SPF stocks of *L. vannamei* that were free of IHHNV. High health offspring from these SPF stocks were made available to U.S. shrimp farmers, resulting in a significant increase in U.S. farmed shrimp production from 1992 - 1994. However, in mid-1995, Taura syndrome virus (TSV) was identified in south Texas, the major shrimp farming region in the U.S., and the resulting TSV epizootic contributed to a 164% decline in Texas shrimp production from 1994 to 1995. USMSFP researchers responded by initiating a selective breeding program to develop TSV-resistant *L. vannamei*. The use of these high-health SPR stocks, in conjunction with on-farm biosecurity practices, resulted in incremental increases in U.S. shrimp production from 1998 to the present. Although TSV-resistant shrimp improved production and profitability for those farmers who were experiencing crop losses from TSV, breeding shrimp for resistance to a single viral pathogen, using current selective breeding strategies, may not be the most effective course of action for the long-term viability of the shrimp farming industry. USMSFP researchers are now developing biosecure shrimp production systems which rely on pathogen exclusion, and research results indicate that it is possible to produce > 5 kg of market-sized shrimp (~ 20 g) per m^2 of raceway in about 12 weeks, using < 400 L of water per kg of shrimp. With advanced biosecure technologies available, the U.S. shrimp farming industry will be able to expand into areas away from the coast with greater control against the spread of disease and without adversely affecting the environment.

Introduction

Shrimp aquaculture expanded significantly during the 1980s and now represents a multi-billion dollar a year industry. In 2002, the global shrimp farming industry produced an estimated 1.6 million metric tons of shrimp, and production is projected to increase at a rate of 12-15% per year over the next several years (Rosenberry 2003). Although farmed shrimp now represent about 50% of the global penaeid shrimp supply, farmers have suffered significant economic losses over the last decade, largely from viral diseases that have plagued the industry (**Table 1**). In Asia, mortalities of cultured shrimp due to White spot syndrome virus (WSSV) and Yellow head virus (YHV) have resulted in significant economic losses (Flegel and Alday-Sanz 1998), and Taura syndrome virus (TSV) is now spreading throughout this region. Similarly, in the Western Hemisphere, both WSSV and TSV have caused catastrophic losses on shrimp farms (Lightner 2003). In Ecuador alone, WSSV was responsible for an estimated 53% decline in shrimp production from 1998 to 2000, resulting in a loss of export revenue in excess of $516 million (Rosenberry 2000).

Virus	Year of Emergence to 2001	Estimated loss
WSSV - Asia	1992	$4 – 6 billion
WSSV - Americas	1999	> $1 billion
TSV	1991 – 1992	$1 – 2 billion
YHV	1991	$0.1 – 0.5 billion
IHHNV[a]	1981	$0.5 – 1.0 billion

[a] Includes Gulf of California fishery losses for 1989 – 1994.

Table 1. Estimated economic losses (in US$) since the emergence of certain viral pathogens in penaeid shrimp aquaculture (modified from Lightner 2003).

In response to these viral pathogens, the global shrimp farming industry is changing the way shrimp aquaculture is practiced. In the United States (U.S.), researchers from the U.S. Marine Shrimp Farming Program (USMSFP) have developed novel approaches to mitigate the impact of shrimp viruses on domestic farm production. USMSFP member institutions involved in this effort include The Oceanic Institute (OI, Waimanalo, Hawaii), University of Arizona (UAZ, Tucson, Arizona), University of Southern Mississippi, Gulf Coast Research Laboratory (Ocean Springs, Mississippi), Waddell Mariculture Research Center (Bluffton, South Carolina), Texas A&M University (Port Aransas, Texas), and Tufts University (Boston, Massachusetts). Several of the approaches developed by the USMSFP have been used successfully in other meat-producing industries, and include the use of specific pathogen free (SPF) and specific pathogen resistant (SPR) stocks, as well as the establishment of biosecure production systems that rely on pathogen exclusion. Importantly, these approaches have evolved over the past decade in response to changing disease problems faced by U.S. shrimp farmers, and their evolution represents an interesting case study on the maturation of an important industry.

Pre-SPF Era for the U.S. Shrimp Farming Industry (1980's – 1991)

Although the first commercial shrimp farm in the U.S. was established in 1967, it wasn't until the late 1980's and early 1990s when the industry began to expand (Rosenberry

2003). During that time, the most commonly cultured species was the Pacific white shrimp, *Litopenaeus vannamei*, because it was considered to be highly resistant to Infectious hypodermal and hematopoietic necrosis virus (IHHNV), a member of the Parvoviridae family (Bonami et al. 1990). IHHNV was first recognized in 1981 when it was associated with catastrophic losses of cultured blue shrimp, *Litopenaeus stylirostris*, in Latin America (Lightner 1999). Despite the relative resistance of *L. vannamei* to IHHNV, shrimp farmers in the Western Hemisphere observed reduced growth rates and cuticular deformities in *L. vannamei* infected with IHHNV. This condition is referred to as Runt Deformity Syndrome (RDS), and RDS represented an economically significant and frequent disease problem of cultured *L. vannamei* (Kalagayan et al. 1991). As much as 30% of cultured *L. vannamei* infected with IHHNV exhibited RDS, and this reduced the market price of IHHNV-infected shrimp by 10-50% relative to IHHNV-free shrimp.

Establishment and Benefits of SPF Shrimp (1991 – 1994)

The increasing incidence of RDS on commercial farms in the U.S. catalyzed USMSFP researchers to develop SPF stocks of *L. vannamei* that were free of IHHNV. Although the SPF concept was well established in other meat-producing industries (Zavala 2001), it had not yet been applied to shrimp. Guidelines for establishing SPF shrimp came from The International Council for the Exploration of the Sea's (ICES) "Code of Practice to Reduce the Risks of Adverse Effects Arising from the Introduction of Non-indigenous Marine Species" (Sindermann 1990). Modifications of the ICES guidelines were used to develop the first SPF stock of penaeid shrimp for the U.S. shrimp farming industry from 1989-1991 (Wyban et al. 1993, Pruder 1994, Pruder et al. 1995). The guidelines stipulate that only disease-causing organisms that can be reliably diagnosed and physically excluded from a facility can be considered in an SPF program. The working list of specific pathogens for SPF penaeid shrimp in the U.S. has changed over time, as new pathogens have been identified and as more advanced disease diagnostic tools have become available. The current SPF list for the U.S. includes eight viruses, one prokaryote, and certain classes of parasitic protozoa (**Table 2**).

Pathogen Type	Pathogen/Pathogen Group	Pathogen Category[2]
Viruses	WSSV – nimavirus (new family)	C-1
	YHV, GAV, LOV – roniviruses (new family)	C-1
	TSV – picornavirus	C-1
	BP – occluded enteric baculovirus	C-2
	MBV –occluded enteric baculovirus	C-2
	BMN – nonoccluded enteric baculovirus	C-2
	IHHNV –systemic parvovirus	C-2
	HPV – enteric parvovirus	C-2
Procaryote	NHP – α-proteobacteria	C-2
Protozoa	Microsporidians	C-2
	Haplosporidians	C-2
	Gregarines	C-3

Table 2. A working list of "specific" and excludable pathogens for penaeid shrimp.[1]

To develop an SPF stock, shrimp are collected from the wild and transferred to a primary quarantine facility where they are analyzed for specifically listed pathogens using appropriate disease diagnostic tools (**Fig. 1**). If shrimp test positive for any of the listed pathogens, they are destroyed in the primary quarantine facility. If shrimp test negative for specifically listed pathogens after several successive screenings, they are transferred to a secondary quarantine facility where they are matured and spawned to produce an F_1 generation of captive shrimp. Because some viruses can be transmitted from parent to offspring (vertical transmission), representative shrimp from the F_1 generation are analyzed for specifically listed pathogens. If shrimp from the F_1 generation test negative for specifically listed pathogens after several successive screenings, they are transferred out of the secondary quarantine facility and can be included as part of the germplasm in a nucleus breeding center (NBC). Shrimp that are maintained in a well-established NBC (i.e. where there is a history of negative disease status documented through a surveillance program) may be designated as SPF (Lotz 1997). However, once shrimp leave an SPF-NBC, they no longer are referred to as SPF even though they may be free of specifically listed pathogens. If shrimp are transferred from an SPF-NBC to a medium-biosecurity facility, their new designation is High Health (HH), indicating that these shrimp are at greater risk of pathogen exposure and infection. If shrimp are transferred to a low-biosecurity shrimp farm, they have entered the Commodity Production (CP) stream, which is most vulnerable to pathogen outbreaks, and the shrimp are neither SPF nor HH.

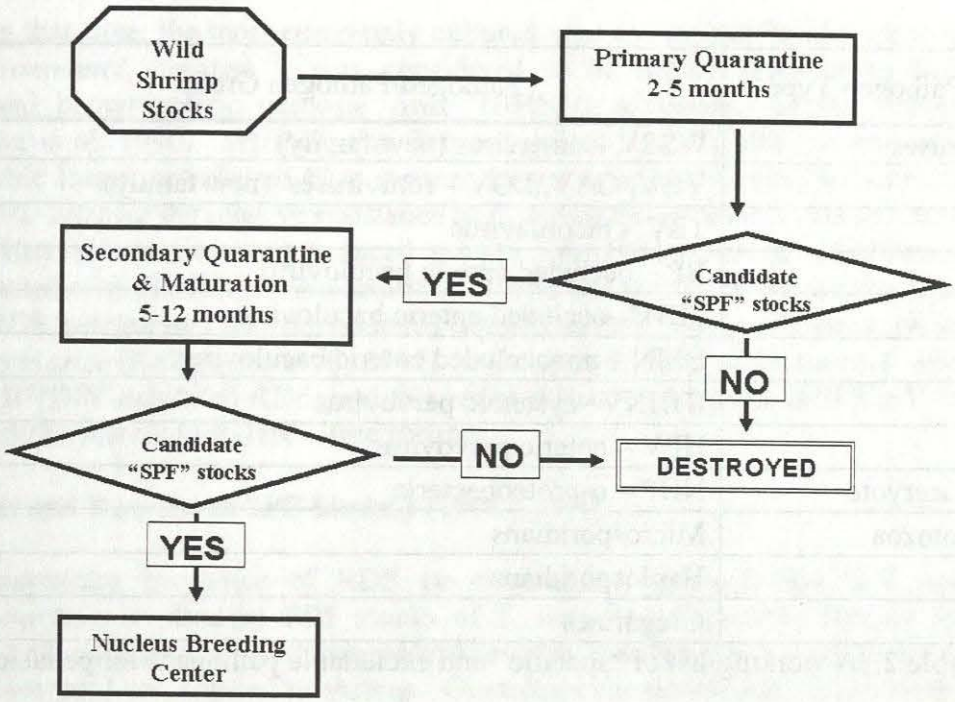

Figure 1. Procedures used to develop specific pathogen free (SPF) shrimp collected from the wild.

Initial growout trials using HH *L. vannamei* indicated that these stocks out-performed non-HH stocks when evaluated at commercial shrimp farms in the U.S. (Wyban et al. 1993). In Texas, Jaenike et al. (1992) reported that HH shrimp obtained from the USMSFP produced a greater yield, higher survival, a more uniform size distribution, and a lower feed conversion ratio than non-HH shrimp. In Hawaii, Carpenter and Brock (1992) reported that HH shrimp produced a greater yield and higher survival than non-HH shrimp when cultured under semi-intensive and intensive culture conditions. Importantly, the HH crop yielded a 62.5% higher return than non-HH crops, and similar improvements were reported in South Carolina (Wyban et al. 1993). The overall effect of using HH shrimp in the U.S. was a significant increase in production from 1992-1994 (**Fig. 2**). This huge impact was most apparent in Texas where the majority of domestic shrimp farming occurs. During this time, production increased from 1.66 million pounds in 1991 to 3.8 million pounds in 1992 and 4.2 million pounds in 1993 (Rosenberry 2003), and this represents a 153% increase in production over two years.

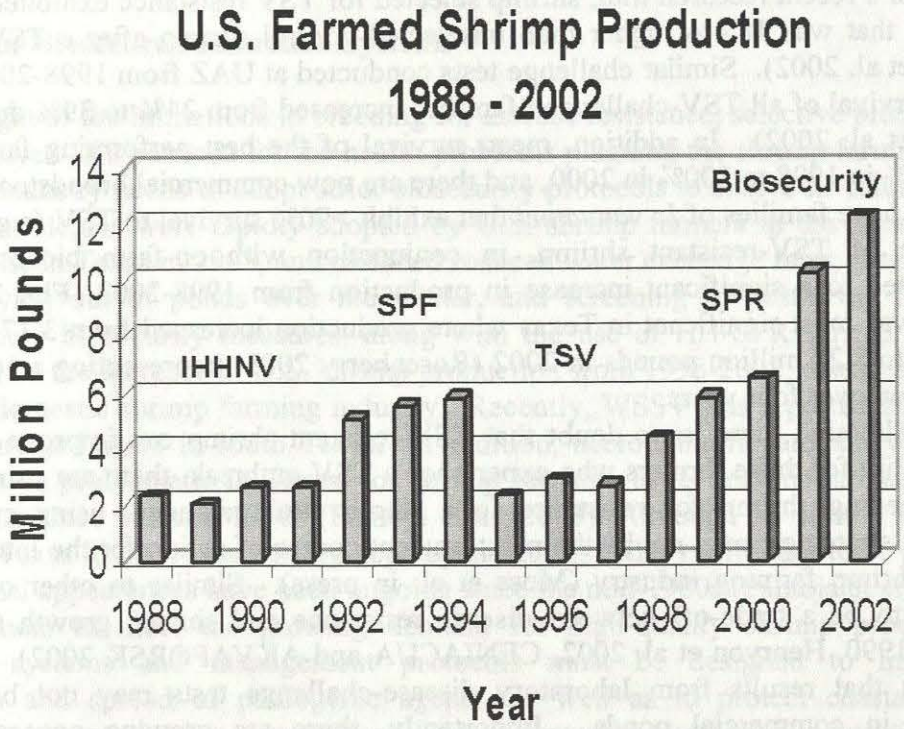

Figure 2. U.S. farmed shrimp production from 1988 – 2002.

Emergence and Impact of TSV in the U.S. (1995 – 1998)

Despite these encouraging results, HH shrimp were not a panacea for the disease problems plaguing the shrimp farming industry (Pruder 1994). In 1993, HH shrimp were cultured with wild-caught seed at a commercial shrimp farm near Rio Guayas in Ecuador. HH shrimp exhibited poor survival (7-43%) compared to wild seed (36-42%), and heavy mortalities were attributed to TSV infection. From this experience, it was demonstrated that HH shrimp cultured in environments with disease problems may not perform well. In mid-1995, TSV was identified in south Texas and the presence of this virus resulted in a significant decline in U.S. farmed shrimp production (Brock et al. 1997, **Fig. 2**). In Texas alone, shrimp production went from 3.69 million pounds in 1994 to 1.4 million pounds in 1995, a 164% decline in one year. Although shrimp production increased from 1995-1998, production levels never exceeded the pre-TSV years when SPF or HH shrimp were available.

Breeding of SPR Shrimp (1998 – present)

In response to the devastating effects of TSV on cultured shrimp in the U.S., USMSFP researchers initiated a selective breeding program to develop a TSV-resistant strain of *L. vannamei*. This approach seemed reasonable, especially in light of the tremendous improvements made through selective breeding of commercially important agriculture crops and animals (see Boyle 1999 for a review on the benefits of chicken breeding). Based on research conducted at OI since 1995, there appears to be additive genetic variation for resistance to TSV in *L. vannamei*, and significant improvements in TSV resistance have been

made. In a recent research trial, shrimp selected for TSV resistance exhibited a mean family survival that was 18.4% higher than unselected control shrimp after a TSV-challenge test (Argue et al. 2002). Similar challenge tests conducted at UAZ from 1998-2000 revealed that mean survival of all TSV-challenged families increased from 24% to 39% during this period (White et al. 2002). In addition, mean survival of the best performing families increased from 65% in 1998 to 100% in 2000, and there are now commercial broodstock suppliers who claim to have families of *L. vannamei* that exhibit >90% survival to TSV (e.g. Wyban 2000). The use of TSV-resistant shrimp, in conjunction with on-farm biosecurity practices, contributed to a significant increase in production from 1998-2002 (**Fig. 2**). Again, this impact was most significant in Texas where production increased from 3.17 million pounds in 1998 to 8.27 million pounds in 2002 (Rosenberry 2003), representing a 161% increase in production over four years.

Although there is no doubt that TSV-resistant shrimp can improve production and profitability for those farmers who experience a TSV outbreak, there are compelling reasons why breeding shrimp for resistance to a single viral pathogen, using current selective breeding strategies, may not be the most prudent course of action for the long-term viability of the shrimp farming industry (Moss et al. in press). Similar to other organisms, there appears to be a trade-off between disease resistance and shrimp growth (Chevassus and Dorson 1990, Henryon et al. 2002, CENIACUA and AKVAFORSK 2002). Also, there are concerns that results from laboratory disease-challenge tests may not be predictive of survival in commercial ponds. Importantly, there are growing concerns about viral mutations, whereby previously resistant shrimp strains may become susceptible to evolving viruses. In fact, this situation occurred recently with TSV.

In 2001, significant mortalities of *L. vannamei* occurred at shrimp farms in Belize resulting from TSV epizootics (Rosenberry 2001), and there were concerns that a new TSV strain had emerged. Researchers from UAZ compared a TSV isolate from Belize with the reference isolate from Hawaii to identify possible differences, using selected OIE (Office of International de Epizooties) diagnostic methods and sequence analysis of nucleotides and amino acids in the viral genome. These researchers concluded that the two isolates exhibited different characteristics and thus represented different strains of the virus (Erickson et al. 2004). Importantly, broodstock suppliers to Belize reported that shrimp bred for resistance to the "old" Taura strain (Hawaii isolate) succumbed to the "new" Belize strain. In response to these concerns, researchers from OI and UAZ explored the possibility of developing selectively bred families of *L. vannamei* that exhibited resistance both to the Hawaii and Belize TSV strains. In a recent trial, several shrimp families from OI's germplasm were identified as having high survival to the Belize strain, and offspring from these families were produced and distributed to U.S. broodstock suppliers and subsequently challenged with both TSV strains (Moss et al. 2003). Selectively bred shrimp exhibited 95% survival after exposure to the Hawaii TSV. This was 75% higher than unselected control shrimp, which exhibited 20% survival after exposure to the same virus. Importantly, selected shrimp exhibited 63% survival after exposure to the Belize TSV, whereas all of the control shrimp died by day 4 of the challenge test. These results indicate that the Belize strain of TSV was more lethal than the Hawaii TSV, although it is possible to develop lines of shrimp that exhibit some resistance to both virus strains.

The Need for Biosecure Production Systems

In light of the limitations in breeding for disease resistance, selective breeding should not be perceived as a panacea for the health problems plaguing the shrimp farming industry. Rather, the industry needs to adopt strict biosecurity protocols to ensure its future. On-farm biosecurity strategies were rapidly adopted by U.S. shrimp farmers in the aftermath of the TSV epizootic in Texas in 1995, and included reduced water exchange rates, filtering of pond influent, drying out of ponds over the winter, and screening of postlarvae for diseases. Although these biosecurity measures, along with the use of HH-SPR shrimp, contributed significantly to the increase in U.S. shrimp production from 1998-2002, diseases continue to impact the domestic shrimp farming industry. Recently, WSSV was reported in Hawaii and TSV has emerged again in south Texas. In addition, necrotizing hepatopancreatitis (NHP) continues to be problematic in Texas, for shrimp farmers, as its seasonal appearances have been ongoing since the mid-1980s (Pantoja et al. 2003). Although no shrimp viruses were detected in Texas in 2002, necrotizing hepatopancreatitis (NHP) continues to be problematic, as its seasonal appearances have been ongoing since the mid-1980s (Pantoja et al. 2003). For shrimp farmers to meet the growing demand for high-quality shrimp products, novel production systems and management protocols must be designed to minimize the introduction and spread of pathogenic agents, as well as to protect coastal resources. Biosecure shrimp production systems represent an emerging alternative to traditional shrimp culture, and provide a high degree of pathogen exclusion with minimal water exchange. In an effort to develop biosecure technologies for the U.S. shrimp farming industry, several researchers from the USMSFP are evaluating prototype systems that may have commercial application (Browdy and Bratvold 1998, Moss et al. 1998, Ogle and Lotz 1998, Samocha and Lawrence 1998).

OI's system consists of a concrete 58-m^2 raceway that is filled with seawater (34 ppt) from an underground aquifer to a depth of about 60 cm. A 2-HP, aspirator-type aerator is used to provide aeration and to move water in a circular pattern around a central baffle. Water flow produces a scouring velocity to keep solids in suspension. For filtration, a 25-ft^3 propeller-washed bead filter is used for solids removal and biological filtration (Malone et al. 1998). The bead filter allows a sufficient amount of microalgae and other suspended particles to pass through so a "green water" environment was maintained. This is important because shrimp reared in water with high concentrations of microalgae and microbial-detrital aggregates grow better than shrimp reared in clean, filtered seawater (Moss 2002). Clear, plastic sheeting (6 mil) is used to cover the raceway as a biosecurity feature to reduce pathogen introduction by airborne vectors. The cover also serves as an effective thermal insulator to maintain desirable water temperatures.

Shrimp production data from this system are encouraging. In a recent trial, juvenile shrimp were stocked at a density of 300/m^2 and were grown to a harvest weight of 19.9 g in 12 weeks (Moss et al. 2002). During this trial, shrimp growth rate was 1.47 g/wk, survival was 86.3%, and production was 5.2 kg/m^2 (52,000 kg/ha/crop equivalent). Importantly, the amount of water used to produce one kg of whole shrimp was about 352 L, and this is two to three orders of magnitude less than what is commonly used by the existing shrimp farming industry. In 1992, Hopkins and Villalón reported the volume of water used by farmers to produce one kg of whole shrimp ranged from 39,000 to 199,000 L. Information used to

determine these values came largely from semi-intensive shrimp farms where liberal water exchange was a common management protocol. Over the past decade, the global shrimp farming industry has made a concerted effort to reduce the amount of water used during shrimp growout. The primary impetus for this change came from efforts to mitigate the introduction of pathogens into the shrimp culture environment, although the collateral benefits of reduced effluent discharge also were recognized. Over the past several years, research institutions and commercial shrimp farms have evaluated intensive shrimp production systems that rely on reduced or zero-water exchange and results indicate that it is possible to reduce significantly the amount of water used to culture shrimp (**Table 3**).

Table 3. Amount of water used to produce one kilogram of whole shrimp. Data are from research institutions and commercial shrimp farms that culture shrimp under intensive conditions and rely on reduced or zero-water exchange.

Species	Water Exchange (%/Day)	Stocking Density (Shrimp/m^2)	Water Use (L/kg shrimp)	Reference
L. setiferus	25.0	40	64,000	Hopkins et al. (1993)
L. setiferus	2.5	40	9,000	Hopkins et al. (1993)
L. setiferus	0	20	6,000	Hopkins et al. (1993)
L. vannamei	0	63-121	2,000	Fast & Menasveta (2000)
L. vannamei	< 10.0	35	1,500	Hamper (2000)
L. vannamei	< 0.5	100	483	Moss et al. (2002)
L. vannamei	< 0.5	200	370	Moss et al. (2002)
L. vannamei	< 0.5	300	352	Moss et al. (2002)

Conclusions

Results from USMSFP research indicate that it is possible to produce > 5 kg/m^2 of market-sized shrimp (~ 20 g) in a biosecure production system in about 12 weeks, using < 400 L of water per kg of shrimp. Although these results are encouraging, the application of this capital-intensive technology only makes sense if shrimp can be produced at a competitive price. Unfortunately, from June 2000 to June 2003, the Urner Barry shell-on white shrimp index dropped from US$6.50 per pound to less than US$3.50 per pound (Rosenberry 2003), thus making it very difficult for U.S. shrimp farmers to make a profit. The good news is that per capita shrimp consumption in the U.S. reached a record level in 2002 to 3.7 pounds. It is hoped that, with advanced biosecure technologies available, the U.S. shrimp farming industry will be able to meet these growing market needs by providing consumers with a high-quality product at a competitive price. Such technologies will allow

shrimp farmers to expand shrimp production into areas away from the coast with greater control against the spread of disease and without adversely affecting the environment.

Acknowledgments

We thank the Shrimp Program at the Oceanic Institute for their technical assistance and commitment to excellence. USDA/CSREES Grant No. 99-38808-7431 awarded to the U.S. Marine Shrimp Farming Program supported this research.

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Trophic Value of the Unicellular Diatom *Phaeodactylum tricornutum* for Larvae of Kuruma Prawn, *Penaeus japonicus*

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Key words: *Phaeodactylum tricornutum*, *Chaetoceros gracilis*, *Penaeus japonicus*, trophic value, larval rearing experiment, large-scale culture, larval production

Abstract

The trophic value of the unicellular and silica-less diatom, *Phaeodactylum tricornutum* for the larvae of kuruma prawn, *Penaeus japonicus,* was evaluated in uni-algal culture, nutritive analyses and larval rearing in this experiment. The algal strain of *Ph. tricornutum* used in this experiment had been selected by more than 100 repeat colony screenings using an agar medium under 30°and light conditions of 50-80 mol/m^2/s. From the results of this repeat selection, the alga grew well at temperatures from 25° to 30°. The limiting temperature and salinity conditions for its growth were from 15° o 35° and from 10 to 40ppt, respectively. Thus, the alga was eurythermal and euryhaline. The optimal reagent medium modified Guillard F medium was Guillard 4F medium without vitamins and silica. Moreover, fertilizers could be used instead of reagents. The algal growth and stationary phases continued for more than 21 days under temperatures lower than 30°. Therefore, the alga was found to be appropriate for culture easily using outdoor, large-scale culture tanks.

The feeding experiment showed that the survival rate of *P. japonicus* zoeal and mysis larvae fed with *Ph. tricornutum* approximated that of larvae fed with *Chaetoceros gracilis,* which is extensively used as a nutritive food organism in the rearing of prawn larvae in Japan. Moreover, the progress of metamorphosis of larvae fed with *Ph. tricornutum* was faster than that of larvae fed with *Ch. gracilis*. In the case of mixed feeding of both algae, the progress of metamorphosis was fast and the survival rate significantly increased in comparison with the sole feeding of each alga. Moreover, *Ph. tricornutum* contains much eicosapentaenoic acid (EPA), crude protein and total lipid. So, the algal nutritive value is evaluated as high. Therefore, *Ph. tricornutum* can be used as a good live food for the larval production of *P. japonicus*.

Introduction

The larval rearing techniques for the kuruma prawn, *Penaeus japonicus*, have been developed actively in Japan. During the spawning season, from June to August, two or three times hatchery cycles are usually carried out. However, the survival rate of prawn larvae at the zoeal and mysis stages is unstable. This uncertainty of production has been attributed to low quality eggs, deterioration of water quality (Okauchi *et al*., 1995; Chuntapa *et al*., 2003), and insufficient feeding. The diatom, *Chaetoceros gracilis*, is extensively used as a nutritive food organism in the rearing of prawn larvae (Chu, 1989). Moreover, the high-temperature tolerant strain has been selected by colony screening (Okauchi, 2002), and some private companies have developed algal automated production systems. Stable algae production has been realized in Japan, and the demand for algae has increased. *Chaetoceros* sp. Concentrate has come onto the market recently from some private companies, but it is too expensive

(about $160 US dollars per 20 liters at a density of about 50 million cells/mL) for most users, and its long-term preservation under refrigeration beyond one month in a refrigerator is difficult. The culture of additional species of algae for prawn larvae is important to increase larval shrimp productivity. Moreover, it was reported that the mixed feeding of several algae species is effective for the improvement of survival and growth rates of four larval shrimp species (Kuban *et al.*, 1985). *Phaeodactylum tricornutum* does not have a siliaceous valve and is a unicellular diatom. Its reproductive and gliding movement are described well (Lewin, 1958). The large-scale cultivation (Ansell *et al.*, 1963) and fatty acid component of the alga (Reitan *et al.*, 1994) were already reported, however, the alga is not commonly used as live food of invertebrate larvae. The alga is used as a live food for artemia, *Artemia salina*, and rotifers, *Brachionus plicatilis* and *B. rotundiformis*, to enrich their eicosapentaenoic acid (EPA) content. If the high-density culture techniques were developed for *Ph. tricornutum* and the nutritive value was evaluated as high, this species could be used as a live food for shrimp larvae like *Ch. gracilis*. Therefore, the algal growth rate and productivity were investigated under various temperature and salinity conditions, and the optimal media, using reagents or fertilizers, were investigated by uni-algal and axenic culture experiments. The effect of mixed feeding of both *Ph. tricornutum* and *Ch. gracilis* was evaluated and compared to the sole feeding of these algae.

Materials and Methods

Alga

The *Phaeodactylum tricornutum* used in this study was received from Dr. Takano in Tokai Regional Fisheries Research Laboratory in 1987. After that, colonies were cultured under temperatures of 30° and 20° in continuous light condition of (50-80μmol/m^2/s). During the past 15 year period, colonies held under each temperature condition were screened using an agar medium more than 100 times. Hereafter, the strains selected under 30° or 20° are designated high-temperature selected strain (H-strain) and low-temperature selected strain (L-strain), respectively. The alga was sterilized using antibiotics.

Uni-algal culture experiment in various temperature conditions

Both H-strain and L-strain of *Ph. tricornutum* were cultured at 5 temperature conditions (15, 20, 25, 30, 35°) using 100mL flat-bottom flasks with 100mL Guillard F medium (Guillard and Ryther, 1962). The culture was batch style, and was carried out under continuous light (80μmol/m^2/s) with sufficient air supply for 21 days. The cell density was estimated using a Coulter Counter or a hemocytometer on the 7^{th}, 14^{th}, and 21^{st} day after inoculation. Quadruplicate flasks under each temperature were used, and average and standard deviation (SD) were calculated. Assuming that the alga increase logarithmically for 7 days after inoculation, the growth rate (k) was calculated using the formula; k (divisions/day) = 1/7 X Log_2 (N_7/N_0). Where N_7 = cell density at 7^{th} day, and N_0 = cell density at inoculation. Moreover, supposing that its population growth changes from growth phase to stationary phase at about the14^{th} day, the cell density at the 14^{th} day seems to be

approaching the maximum. Decreasing rate (d) was calculated using the formula; d (%/day) = $(N_{21}-N_{14})/7X100$. Where N_{21} = cell density at 21st day, and N_{14} = cell density at 14th day.

Uni-algal culture experiment in various salinity conditions

The H-strain of *Ph. tricornutum* was cultured under 8 step-wise salinity conditions (5, 10, 15, 20, 25, 30, 35, 40ppt). Four 500mL flat-bottom flasks with various salinities of 400mL GuillardF medium, were used in each salinity group. Their salinity was controlled with distilled water or boiled seawater. The culture was continued for 7 days, and the growth rate calculated using the above-mentioned formula.

Uni-algal culture experiment to make an optimum fertilizer medium for large-scale culture

Three trials were conducted. First, the suitable reagent medium was determined by modifying GuillardF medium (Trial-1). Except for silica and vitamins (Vitamin B_{12}, Biotin, and Thiamin Hydrochloride), other nutrients, which composed GuillardF medium, were added at one to ten times the quantity of those into autoclaved seawater. Each medium was abbreviated GuillardF to 10F. Then, the additive amount of each fertilizer required for optimal growth of the alga was investigated in Trial-2. Potassium nitrate (KNO_3), Calcium dihydrogenphosphate ($Ca(H_2PO_4)_2$) and Clewat32, which is metal powder on sale from Teikoku Kagaku Sangyo Co., were selected as fertilizers. As test groups (n = 4 each), H-strain of *Ph. tricolnutum* was cultured for 10 days in seawater with eight step-wise addition of each fertilizer (the quantity of KNO_3; 178.5, 356.8, 535.0, 713.5, 892.0, 1070.5, 1248.8, and 1427.3μg/mL, that of $Ca(H_2PO_4)_2$; 9.2, 18.4, 27.6, 36.8, 46.0, 55.2, 64.4, and 73.6μg/mL, that of Clewat32; 40, 80, 120, 160, 200, 240, 280, and 320μg/mL). The alga was cultured in Guillard4F as a control group (n=4). In Trial-3, effectiveness of the addition of vitamins and silica on the growth of the alga was evaluated. H-strain of the alga was cultured in the optimum reagent and fertilizer media, which were selected in Trial-1 and 2, with or without vitamins and silica.

All cultures were carried out using 500mL flat-bottom flasks with 400mL of each medium in light and temperature controlled room at 60 to 80μmol/m^2/s (24 light) and 25°, respectively. Cultures were continuously aerated at a rate of about 1L/min/vessel. The alga inoculated grew well in the pre-culture using Erd-Schreiber medium. These trials continued for 10 days. An estimate of the cell density of all cultures was made at the end of each trial using a Coulter Counter. The productivities in various media were calculated from the formula; p ($X10^4$ cells/day) = $(N_{10}-N_0)/10$. Where N_{10} = cell density at 10th day, and N_0 = cell density at inoculation. The average productivity and SD (n=4) of a particular group was calculated from the results of quadruplicate vessels.

Feeding experiment to kuruma prawn larvae

Penaeus japonicus nauplii used in this experiment were hatched from eggs obtained from a single female. Some hours after hatching, vigorous nauplii were collected using a pipette and a Petri dish, and were divided into 12 groups of 1,500 larvae each. A group was held in a 5L plastic circular tank containing 5L of autoclaved seawater. Four tanks were used for each treatment. The larvae were reared from nauplii to mysis-3 stage or post larval-1 stage for 14 days by feeding *Ph. tricornutum* only (Test I group; PH-1 to 4), mixed feeding both *Ph. tricornutum* and *Ch. gracilis* (Test II group; PH&CH-1 to 4), or feeding *Ch. gracilis* only (Control group; CH-1 to 4). Initial density of algae in Test I, Test II, and Control groups was $16X10^4$ cells/mL (*Ph. tricornutum*), $8X10^4$ cells/mL (*Ph.tricormutum*) and 10^5 cells/mL (*Ch. gracilis*), and $20X10^4$ cells/mL (*Ch. gracilis*), respectively. Algal cell densities in every tanks were examined daily under a microscope with hematocytometers, and they were maintained at $12\text{-}18X10^4$ cells/mL (*Ph. tricornutum*) in Test I group, $5\text{-}10X10^4$ cells/mL (*Ph. tricornutum*) and $8\text{-}15X10^4$ cells/mL (*Ch. gracilis*) in Test II group, and $15\text{-}22X10^4$ cells/mL (*Ch. gracilis*) in the Control group by either diluting rearing water with seawater or by supplementing cultured algae. Daily estimate of the survival larval number in every tank was made by counting the vigorous swimming larvae in 500mL rearing water using 500mL measuring cylinders and watch glasses, and the average and SD in each group (n=4) were calculated. Moreover, 100 larvae each were randomly sampled from every treatment group and fixed with 1% formalin to determine the larval metamorphosis stage using a stereoscopic microscope. The larval rearing experiment was carried out in a temperature and light controlled room at 25° and 50μmol/m^2/s (14L:10D), and sufficient air was supplied continuously. During the experiment, 1L of rearing water in every tank was drained out and autoclaved fresh seawater was added once a day. *Phaeodactylum tricornutum* and *Ch. gracilis* were cultured using 10L glass carboys in Guillard4F medium.

Contents of crude protein, total lipid, and ash of feeding *Ph. tricornutum* and *Ch. gracilis,* which were randomly collected four times during the larval rearing experiment, were measured using common methods. Moreover, the fatty acids component of total lipid in *Ph. tricornutum*, which were cultured in Guillard4F medium, was analyzed by a gas-liquid chromatography.

Results

Morphological features

Figure 1 shows the *Ph. tricornutum* which have increased vigorously in the growth phase. Most cells are the fusiform, and some cells are trigonometrical and ovoid types. Apical axis and transapical axis of the fusiform cell are 12-18μm and 3-5μm, respectively. Packed cell volume is about 55μm^3. Its chloroplast grows remarkably well and the transapical axis extends in a eutropic medium, however, it shrinks in an oligotropic medium (**Fig.2**).

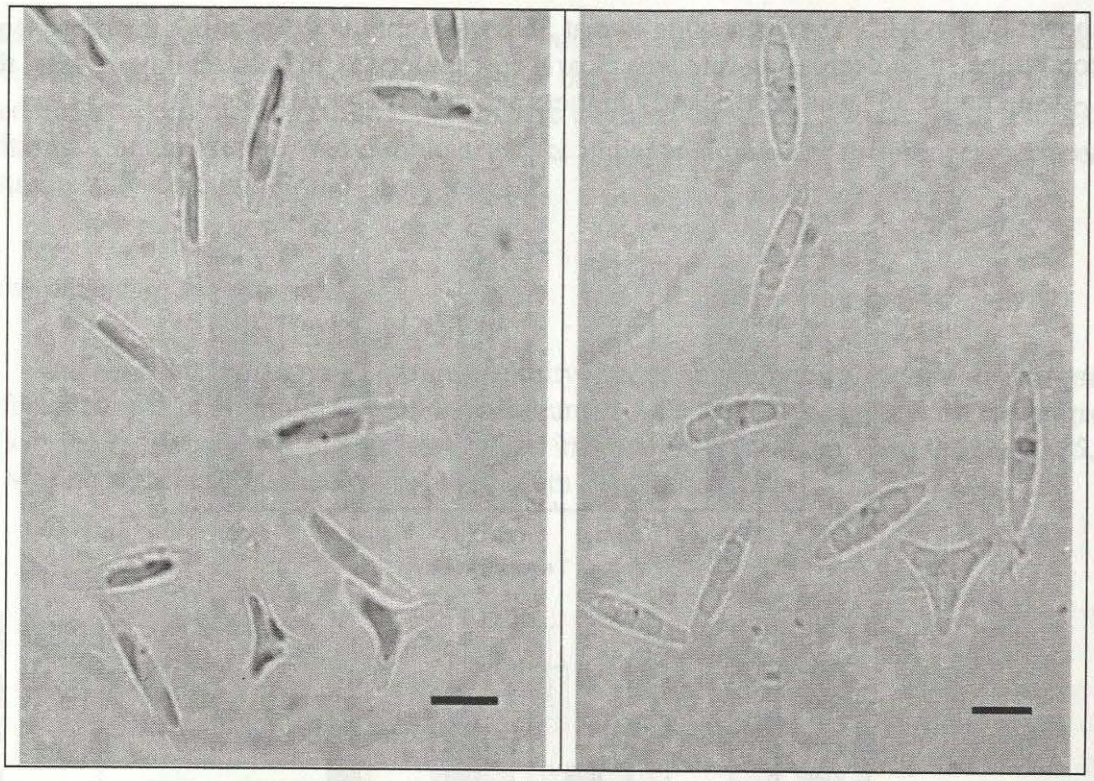

Figure 1 (on the left). *Phaeodactylum tricornutum* increased vigorously in Guillard 4F medium. Most cells are fusiform, and some cells are trigonometrical and ovoid forms. Chloroplasts grew remarkably well. Bar shows 5 m.

Figure 2 (on the right). *Phaeodactylum tricornutum* which increased in an oligotropic medium which lacks some heavy metals. Chloroplast in the middle part of the cell is reduced in size. Bar shows 5 m.

Growth under various temperature

The growth rate of the H-strain, which was selected under 30°, was 0.6 divisions/day under 20° to 30° (25°=30°=20°>15°=35°; $P<0.05$). While, that of L-strain, which was selected under 20°, was 0.6 divisions/day under 20° to 25° (25°=20°>30°=15°>35°; $P<0.05$) (**Fig.3-A**). In comparing both strains, the growth rates of H-strain under 30° and 35° were significantly higher than those of L-strain ($P<0.05$ and $P<0.01$, respectively). The cell density at the 14th day after inoculation is shown in **Fig.3-B**. The cell density of H-strain under 25° and 30° was significantly higher than that under other temperature conditions (25°=30°>20°>15°>35°; $P<0.05$). The density of the L-strain under 25° and 20° was significantly higher than that under other temperature conditions (25°=20°>30°>15°>35°; $P<0.05$). The density of H-strain under 25°, 30° and 35° was significantly higher than that of L-strain ($P<0.05$, $P<0.01$, and $P<0.01$, respectively). Moreover, the decreasing rate, which calculates from the difference between the cell density of 14th day and that of 21st day, is

shown in **Fig.3-C**. The decreasing rate at 35°, was a minus value under the other temperature conditions. The decreasing rate was clearly low under 15° in both strains. While, the rate of L-strain under 35° was significantly higher than that of H-strain ($P<0.01$).

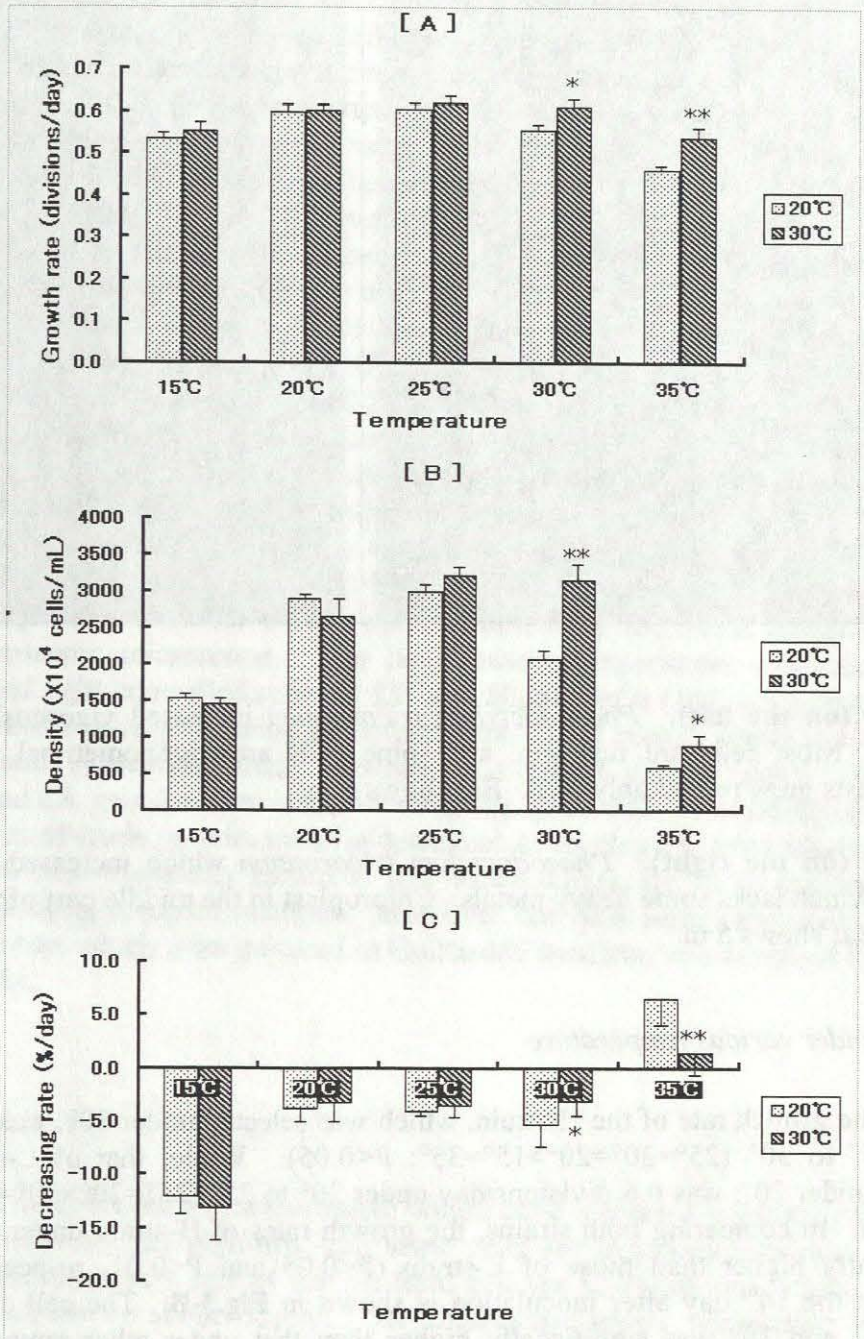

Figure 3. Growth features of the two *Ph. tricornutum* strains that were made by repeat colony selection using agar medium under 20°C or 30°C. Growth rates, which are calculated from the cell densities at inoculation and the 7th day, are shown in [A], cell densities on the 14th day after inoculation are shown in [B], and decreasing rate which are calculated from the difference between cell density on the 14th day and that on the 21st day are shown in [C].

Dotted and obliquely striped bars in [A], [B],and [C] show average values (n=4) of strains selected 20°C and 30°C respectively. Vertical bars indicate SD (n=4). Statistical tests were carried out between the two strains, which were cultured under the five stepwise temperature conditions. Single asterisk and double asterisks indicate significant differences between the two strains at $P<0.05$ and $P<0.01$, respectively.

Growth in various salinity media

The algal growth rates in various salinity media are shown in **Figure 4**. No growth was observed in 5 ppt medium. In 10 ppt medium, the alga could increase with lower growth rate (k = 0.33). While, the algal growth rates in 15 to 40 ppt media were 0.62 to 0.65, and there was no significant difference between them ($P>0.05$).

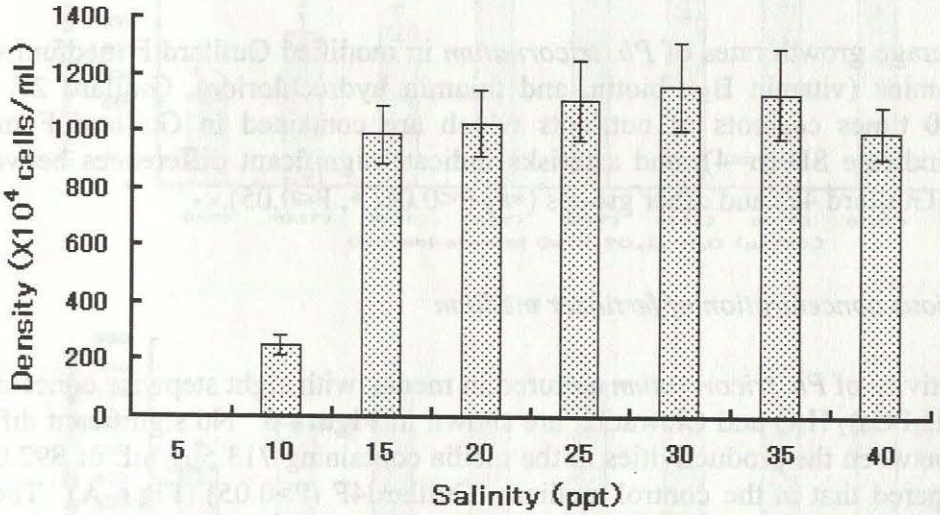

Figure 4. Average growth rates of *Ph. tricornutum* in various salinity media. Vertical bars indicate SD (n=4).

Growth in modified GuillardF medium

The productivity of *Ph. tricornutum* H-strain, which was cultured in GuillardF to 10F medium for 10 days, is shown in **Figure 5**. The value of the alga cultured in Guillard4F, 5F and 6F medium was about $480X10^4$ cells/mL/day and was significantly higher than that in other media except for Guillard8F. From the result of this experiment, Guillard4F was selected as a suitable reagent medium, and it was used as the control medium in Trial-2 and 3.

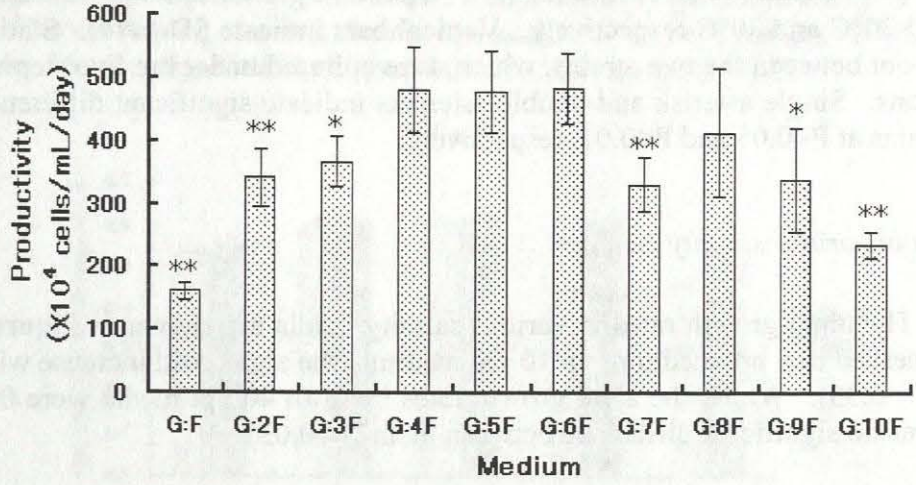

Figure 5. Average growth rates of *Ph. tricornutum* in modified Guillard F medium without silica and vitamins (vitamin B_{12}, biotin, and thiamin hydrochloride). Guillard 2F to 10F means 2 to 10 times contents of nutrients which are contained in Guillard F medium. Vertical bars indicate SD (n=4), and asterisks indicate significant differences between the control group (Guillard 4F) and other groups (**; $P<0.01$, *; $P<0.05$).

Growth in various concentration of fertilizer medium

Productivity of *Ph. tricormutum* cultured in media, with eight stepwise concentrations of KNO_3, $Ca(H_2PO_4)_2\ H_2O$ and Clewat32, are shown in **Figure 6**. No significant difference was detected between the productivities in the media containing 713.5μg/mL or 892.0μg/mL of KNO_3 compared that in the control medium; Guillard4F ($P>0.05$) (**Fig.6-A**). Therefore, the suitable concentration of KNO_3 was considered to be 713.5μg/mL to 892.0μg/mL. The contents of NO_3-N in 713.5μg/mL and 892.0μg/mL of KNO_3 were almost equal those of Guillard4F and 5F media. In case of $Ca(H_2PO_4)_2\ H_2O$, the productivity of the alga in the media that contained 9.2μg/mL to 27.6μg/mL of that fertilizer, were significantly lower than that in control medium; Guillard4F ($P<0.05$). However, the productivity of media containing 36.8μg/mL to 73.6μg/mL of $Ca(H_2PO_4)_2\ H_2O$ were the same as that in control medium ($P>0.05$) (**Fig.6-B**). Therefore, 36.8μg/mL of $Ca(H_2PO_4)_2H_2O$ regarded as a suitable concentration. The content of PO_4-P in 36.8μg/mL of $Ca(H_2PO_4)_2H_2O$ was almost equal with that of Guillard4F medium. While, productivity of the alga in the media with 80μg/mL to 320μg/mL of Clewat32 were almost equal that of Guillard4F as control ($P>0.05$) (**Fig.6-C**). In comparing the productivity of 120μg/mL medium and that of 80μg/mL medium, significant difference was detected between them ($P<0.05$). Thus, the suitable concentration of Clewat32 was 120μg/mL to 320μg/mL.

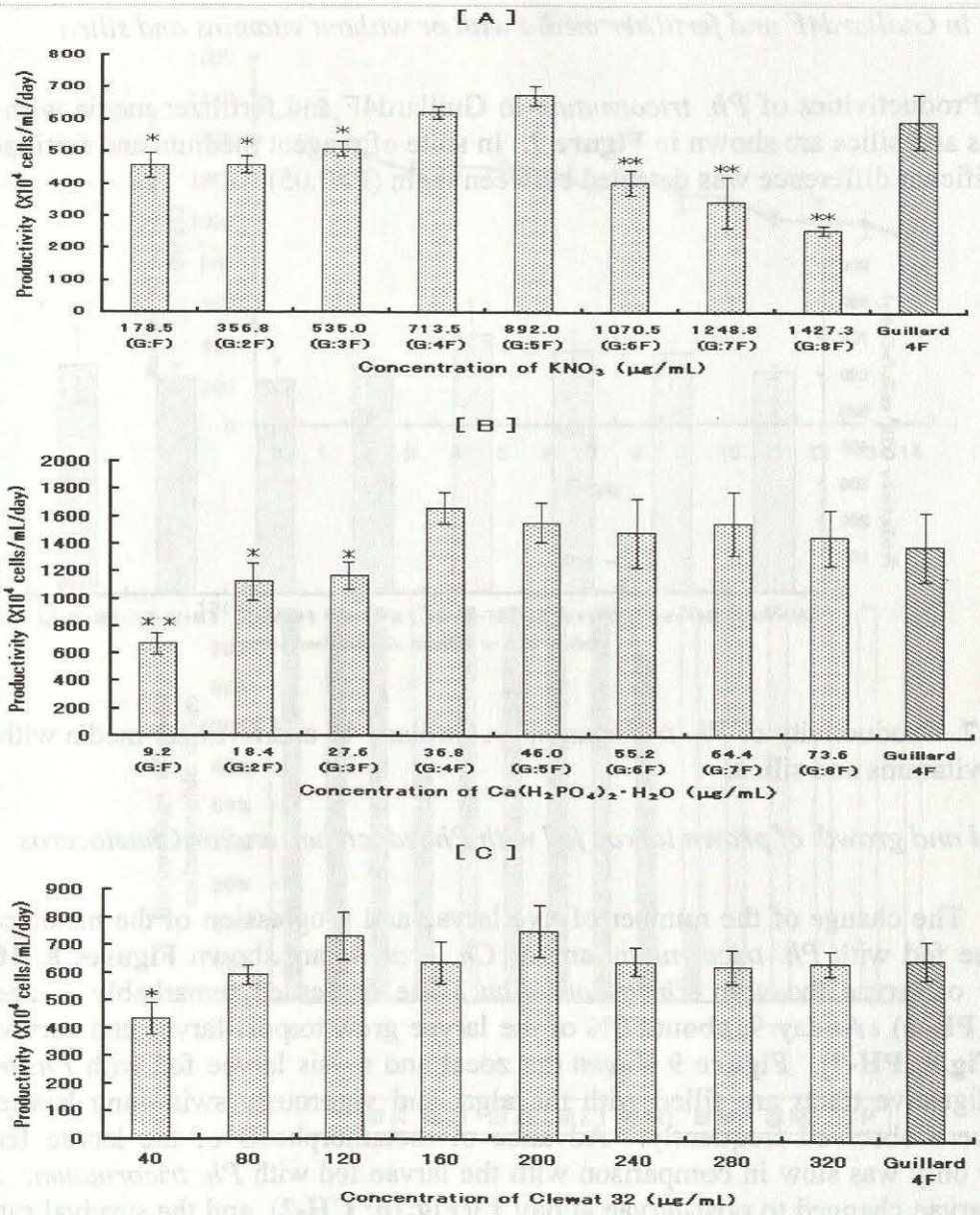

Figure 6. Productivity of *Ph. tricornutum* cultured for ten days in fertilizer media containing various quantities of Potassium nitrate (KNO_3); [A], Calcium dihydrogenphosphate monohydrate ($Ca(H_2PO_4)_2H_2O$); [B], and Clewat 32 (heavy metal compound which is sold from Teikoku Kagaku Sangyo Co.); [C]. Productivity in each fertilizer medium was compared with that in Guillard 4F medium as the control. Marks in parentheses are for Guillard F to Guillard 8F (G:F – G:8F) media which includes the same quantity of NO_3-N or PO_4-P as in the various fertilizer media. Vertical bars indicate SD (n=4), and asterisks indicate significant differences between the control group (Guillard 4F) and others (**; $P<0.01$, *; $P<0.05$).

Growth in Guillard4F and fertilizer media with or without vitamins and silica

Productivities of *Ph. tricornutum* in Guillard4F and fertilizer media with or without vitamins and silica are shown in **Figure 7**. In spite of reagent medium and fertilizer medium, no significant difference was detected between them (P>0.05).

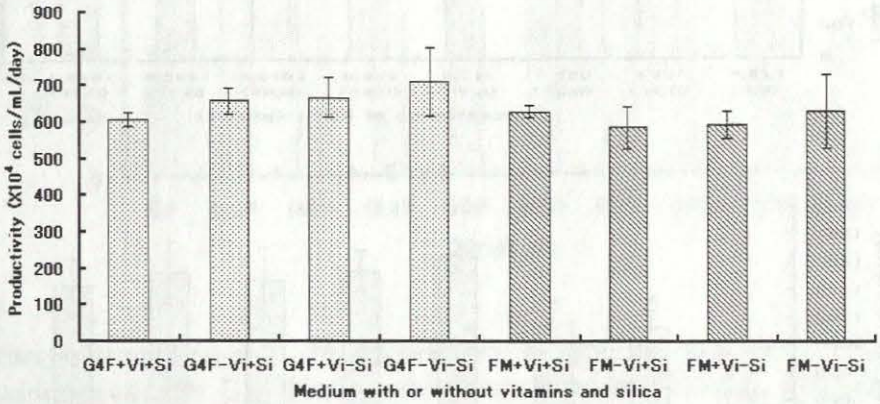

Figure 7. Productivity of *Ph. tricornutum* in Guillard 4F and fertilizer media with or without vitamins and silica.

Survival and growth of prawn larvae fed with Phaedactylum and/or Chaetoceros

The change of the number of live larvae and progression of the metamorphic stage of larvae fed with *Ph. tricornutum* and/or *Ch. gracilis* are shown **Figures 8, 10, 11**. The number of larvae fed with *Ph. tricornutum* alone decreased remarkably at zoeal–1 stage (**Fig.8; PH-1**). At day 9, about 80% of the larvae grew to post-larvae and survival rate was 80% (**Fig.8; PH-2**). **Figure 9** shows the zoeal and mysis larvae fed with *Ph. tricornutum*. Their digestive tracts are filled with the alga, and vigorously swimming larvae with long feces were observed frequently. Advance of metamorphosis of the larvae fed with *Ch. gracilis* only was slow in comparison with the larvae fed with *Ph. tricornutum*. About 80% of the larvae changed to post-larvae at day 13(**Fig.10; CH-2**), and the survival rate at day 13 was 65% (**Fig.10; CH-1**). During zoea-2 stage to mysis-3 stage, the survival rate of the larvae decreased remarkably (**Fig.10; CH-1**). The larvae fed with both *Ph. tricornutum* and *Ch. gracilis* survived well, and their survival rate at day 10, when about 80% of the larvae changed to post-larvae (**Fig.11; PH&CH-2**), was 92% and there was only a small reduction in the survival rate during the experiment (**Fig.11; PH & CH-1**). Moreover, the larvae seemed to eat both algae without selection.

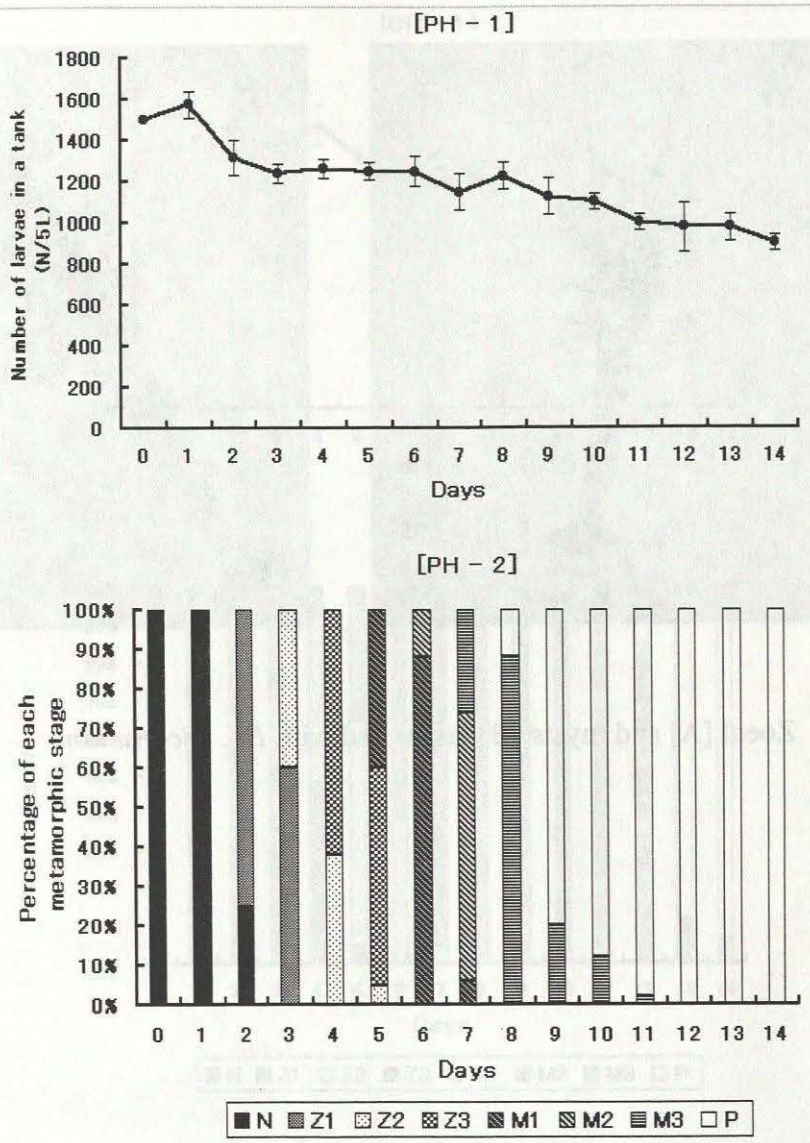

Figure 8. Change in the average number of living larvae, which were fed with *Ph. tricornutum,* in four tanks [PH-1], and the progress of the metamorphic stages of larvae fed with the alga [PH-2]. Vertical bars in [PH-1] indicate SD (n=4), and N, Z1, Z2, Z3, M1, M2, M3, and P means nauplius stage, zoea-1 stage, zoea-2 stage, zoea-3 stage, mysis-1 stage, mysis-2 stage, mysis-3 stage, and post larvae, respectively.

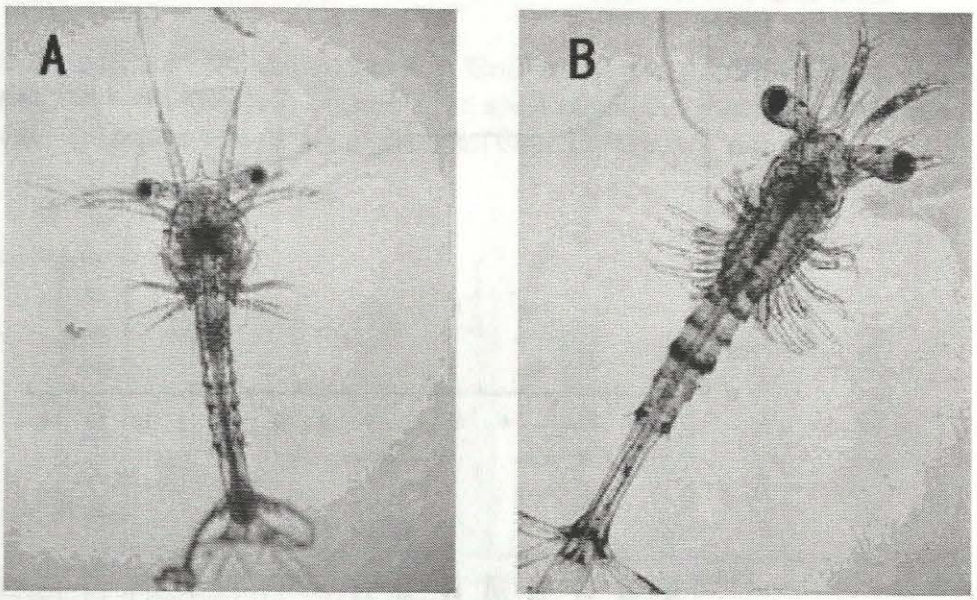

Figure 9. Zoeal [A] and mysis [B] larvae fed with *Ph. tricornutum*

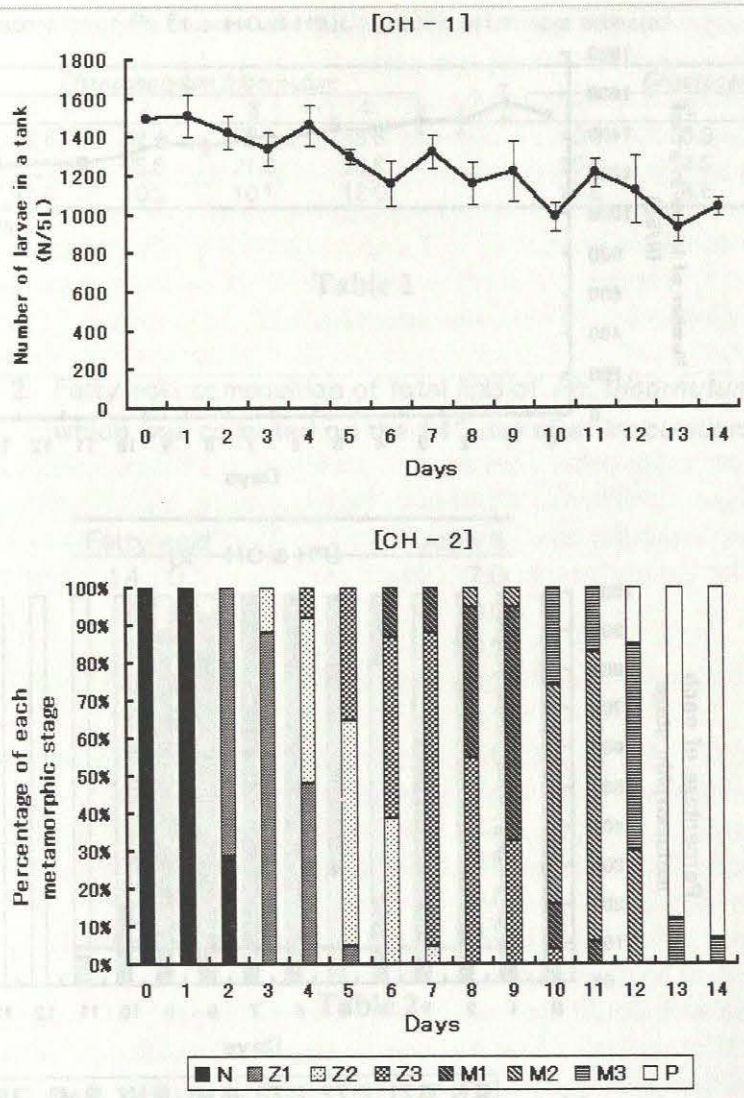

Figure 10. Change of the average number of living larvae, which were fed with *Ch. gracilis* in four tanks [CH-1], and the progress of metamorphic stages of larvae fed with the alga [CH-2]. For further explanation see **Figure 8**.

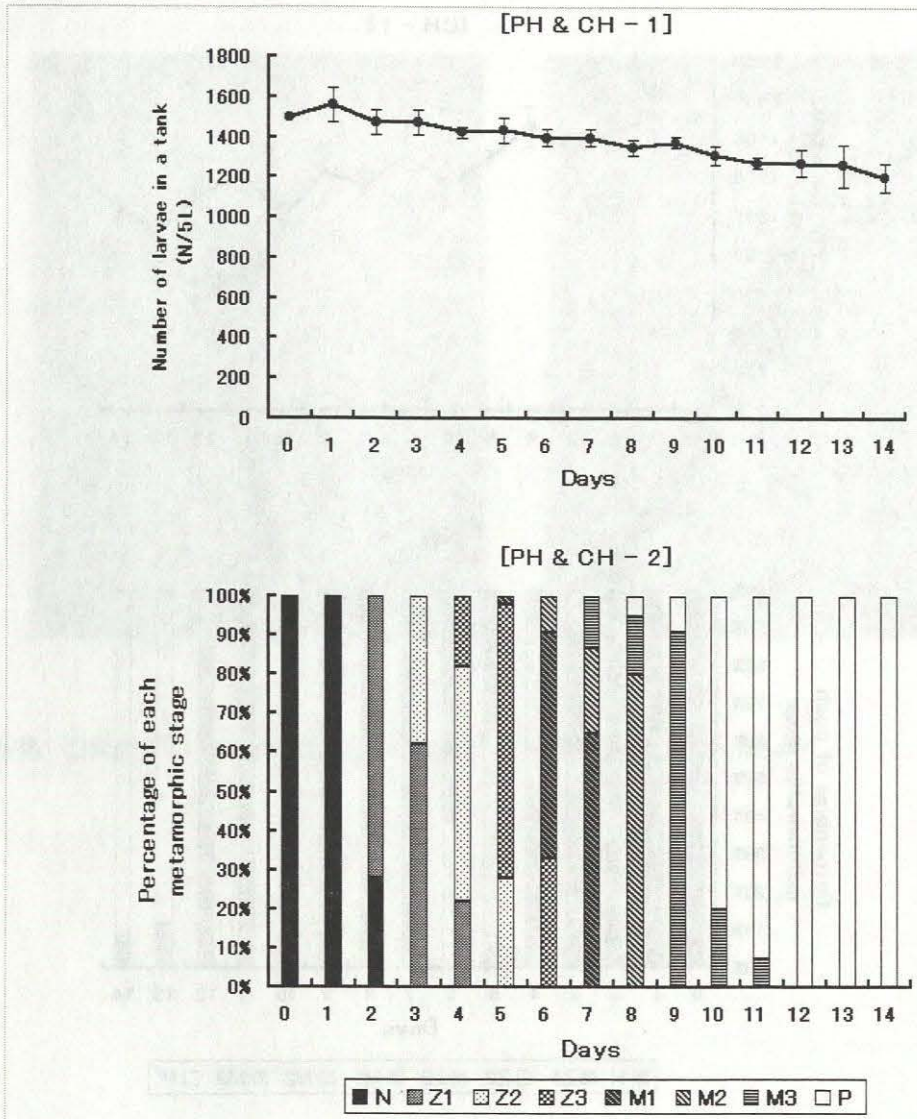

Figure 11. Change of the average number of living larvae, which were fed with both *Ph. tricornutum* and *Ch. gracilis*, in four tanks [PH & CH-1], and the progress of metamorphic stages of larvae fed with both algae [PH & CH-2]. For further explanation see **Figure 8**.

Contents of crude protein, total lipid, and ash in *Ph. tricornutum* compared with those in *Ch. gracilis* are shown in **Table 1**. The average contents of these components in *Ph. tricornutum* were 56.6%, 22.8% and 11.1%, respectively, while those contents in *Ch. gracilis* were 35.7%, 16.2% and 24.0%, respectively. The fatty acid component of total lipid in *Ph. tricornutum* is shown in **Table 2**. The alga contained 12.5% of 20:5 fatty acids.

Table 1. Proximate composition of *Ph. tricornutum* and *Ch. gracilis* which were collected

	Phaeodactylum tricornutum				*Chaetoceros gracilis*			
	1	2	3	4	1	2	3	4
Crude protein *	59.8	62.4	55.3	48.8	40.6	38.8	32.4	31.0
Total lipid *	23.5	25.5	21.8	20.5	15.5	14.5	16.9	17.8
Crude ash *	11.8	10.5	10.1	12.0	27.8	24.6	22.3	21.2

* On a dry matter basis

Table 1

Table 2. Fatty acid composition of total lipid of *Ph. tricornutum* which was collected on the 14th day after inoculation.

Fatty acid	Area %
14 : 0	7.0
14 : 1	0.0
16 : 0	20.5
16 : 1	43.3
16 : 2	3.1
16 : 3	7.6
18 : 0	6.4
18 : 1	2.2
18 : 2	0.0
20 : 4	0.0
20 : 5	9.9

Table 2

Discussion

Characteristics of the growth of Ph. tricornutum H-strain and L-strain

When both strains were cultured under 30° and 35°, the difference of growth characteristics was apparent. The growth rate and the cell density at day 14 of H-strain were significantly higher than those of L-strain. The difference was considered to be induced by the repeat selection for higher temperature tolerance under 30°, and the H-strain would adjust to higher temperature conditions. The difference was minimal between 15° to 25°. Thus, the H-strain can grow well under the low temperature conditions the same as the L-strain, and it is a eurythermal strain. Moreover, the decreasing rates of both strains were negative values between 15° to 30°. The growth rates and the cell density at day 14 for both strains and were remarkably lower under 35° and the decreasing rates were a positive value. This tendency was clearly seen in the L-strain. So the limiting upper temperature was 35°. The negative value for decreasing rate means that the alga continuously increased until day 21 and the stationary phase was long. Therefore, *Ph. tricornutum* could be increased continuously when grown between 15° to 30° for 21 days. Because semi-continuous culture was possible, and

its growth phase continued for a long term, the alga is suitable for mass cultivation as a live food organism.

The salinity condition for good growth of H-strain was from 10 to 40ppt, so that strain is euryhaline, too. This character is desirable as a food organism, because the alga can be cultured in a wide range of salinity, which is appropriate for the good growth of the target culture species. Thus, *Ph. tricornutum* H-strain is suitable as a live food organism, in terms of accommodation to salinity.

Morphological feature of Ph. tricornutum H-strain

As shown in **Figure 1**, the algal cells, which grew in a medium that includes sufficient nutrients, was fusiform, and their prevalence and transapical axes were long. The algal chloroplast grew well in the nutrient medium and its nutritive value estimates are high. Thus, the algal nutritive value can be estimated roughly by observation using a photomicroscope. As the size of apical axis is longer than that of *Chaetoceros*, there was concern whether the prawn larvae could eat them easily.

Suitable reagent medium for Ph. tricornutum H-strain culture

Guillard4F, 5F, and 6F were concluded to be suitable reagent media for the alga because the difference quantities of nutrients are small between these levels of media. Guillard4F was selected as the most suitable medium in this experiment. This medium level is recommended for use in small-scale culture, which is lower than 1,000mL.

Possibility of using fertilizers substitute for reagents to make a suitable medium

If fertilizers could be used as nutrients instead of reagents, the cultivation is economical and easy, especially in outdoor large-scale culture. In this experiment, KNO_3, $Ca(H_2PO_4)_2H_2O$, and Clewat32, which are used agriculture, were selected as N-source, P-source, and heavy metal nutrients source respectively. From the result of Trial-2, it is clear that the alga increased as well in fertilizer medium as it did in reagent medium. The efficiency of Clewat32 was very conspicuous in that the algal chloroplast was well developed.

Moreover, from the result of Trial-3, it was proved that silica and vitamins were not essential nutrients for the good growth of the alga. High levels of silica induces white sediments in seawater, and vitamins are expensive and induce propagation of bacteria. The use of these components should be avoided. *Phaeodactylum tricornutum* does not require them in outdoor large-scale culture or indoor culture.

Food value of Ph. tricornutum to prawn larvae

From the result of larval rearing experiment, *Ph. tricounrutum* is considered to be a suitable food organism for kuruma prawn zoeal and mysis larvae. For larvae fed on *Ch. gracilis* alone, the number of larvae clearly decreased during zoeal-1 and zoeal-2 stages. The

reason seems to be that *Ph. tricornutum* is bigger than *Ch. gracilis*, so that some larvae eating the latter starved to death. After the zoeal-2 stage, the number of larvae fed *Ph. tricornutum* did not decline noticeably. The larvae fed on *Ph. tricornutum* metamorphosed faster than those fed on *Ch. gracilis*. At day 9, about 80% larvae fed on *Ph. tricornutum* alone grew to post larvae, while most larvae fed on *Ch. gracilis* were only at mysis-1 or mysis-2 stage. One of the reasons for the difference in metamorphosis progress was considered to be the nutritive difference between *Ph. tricornutum* and *Ch. gracilis*. *Phaeoductylum tricornutum* used in this experiment contained 20:5 fatty acid. From the results of the algal fatty acid composition reported (Ackman *et al.* 1968, Moreno *et al.* 1979), the 20:5 fatty acid seems to be EPA and fatty acids component of both diatoms are similar. The crude protein and total lipid contents in *Ph. tricornutum* were about 1.2 to 1.5 times higher than those of *Ch. gracilis*, so that the nutritive value of *Ph. tricornutum* was evaluated high compared with *Ch. gracilis*. However, the contents of crude protein and total lipid usually change under various culture conditions using batch style culture (James *et al.* 1989, Okauchi *et al.* 1990). Therefore, more detailed research on these algal nutrient changes relative to culture conditions will be required.

Effect of mixed feeding of Ph. tricornutum and Ch. Gracilis

It is already shown in bivalve larval rearing experiments that mixed feeding of some algae species, whose nutritive values are evaluated as high, is superior to sole feeding of each alga (Davis and Guillard, 1958). In this experiment, the survival rate of the prawn larvae fed on both algae was high compared with that of sole feeding trials. The larval survived in a tank was little changed during the mixed feeding experiment. Moreover, the progress of metamorphosis was almost same as that of larvae fed on *Ph. tricornutum*. Such a mixed feeding of some algae is effective not only for survival and growth of bivalve larvae, but also those of prawn larvae. Kuruma prawn larval rearing is usually carried out during the rainy and hot-temperature season, using outdoor large-scale tanks, in Japan. Under bad weather conditions, it is difficult to prepare enough *Ch. gracilis*. Therefore, culture of some species of algae with high nutritive value and growth capacity, such as *Ph. tricornutum*, *Isochrysis* sp.(Tahiti) (Okauchi et al. 1997), *Tetraselmis tetrathele* (Okauchi and Hirano, 1988), and *Tetraselmis chuii* (Tobias-Qunitio and Villegas, 1982) is important to avoid insufficient food supply. Moreover, the mixed feeding of these algae is effective to stabilize prawn larval production.

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Application of Biosecurity in Aquaculture Production Systems

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Abstract

A significant challenge to the expansion of aquaculture production is the outbreak of disease. Potential economic losses from disease outbreaks are significant, and can affect the survival of the industry. The occurrence of disease is a combination of the health of the animal, the condition of the environment, and the presence of a pathogen. The poultry industry has implemented a biosecure production system to prevent the spread of infectious disease among farms. It serves as a model to aquaculture as a reliable source of animal protein worldwide. This paper briefly highlights some of the major points and practices of biosecurity for various aquaculture production systems presented at a special workshop held in Honolulu in July 2001 and published in the proceedings, "Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables" (see Lee and O'Bryen 2003). Examples of biosecurity systems used domestically and internationally in shrimp farming, finfish culture, and mollusc culture, as well as regulations and policies to prevent and control the spread of aquatic animal diseases are provided. The key elements of biosecurity are a reliable source of stocks, adequate detection and diagnostic methods for excludable diseases, disinfection and pathogen eradication methods, best management practices, and practical and acceptable legislation.

Introduction

Production from aquaculture has grown at an impressive annual rate of approximately 11% since 1980. One of the significant challenges to the expansion of aquaculture production is from disease outbreaks. Diseases caused by viral infection are not easily treated under current technology and have caused significant economic losses. Potential economic losses from disease outbreaks are significant, and can affect the survival of the industry. For example, viral disease outbreaks have caused billions of dollars in lost revenue for the global shrimp industry (Lightner 2003, **Table 1**). Operation of shrimp farming once became impossible in countries such as Ecuador, Taiwan, and China due to disease outbreaks. The most effective way to deal with viral infection is to prevent it from occurring.

The success of the poultry industry as a reliable source of animal protein worldwide has been due to the implementation of a biosecure production system to prevent the spread of infectious disease among farms. The biosecurity practices in the poultry industry have prompted, in recent years, the consideration of a similar practice in aquaculture to deal with disease problems. The lessons learned from the poultry industry will assist the development of biosecurity in aquaculture.

Table 1. Estimated economic losses since the emergence of certain diseases in penaeid shrimp aquaculture.

Virus	Year of emergence to 2001	Product loss (US dollars)
White Spot Syndrome Virus-Asia	1992	$4-6 billion
White Spot Syndrome Virus-Americas	1999	>$1 billion
Taura Syndrome Virus	1991-1992	$1-2 billion
Yellow Head Virus	1991	$0.1-0.5 billion
Infectious Hypodermal and Hematopoietic Necrosis Virus	1981	$0.5-1.0 billion (includes Gulf of California fishery losses for 1989-1994)

Source: Lightner (2003, p. 85)

The National Oceanic and Atmospheric Administration has provided the Aquaculture Interchange Program (AIP) at the Oceanic Institute in Hawaii with funding to gather information related to biosecurity measures used against the spread of bacterial diseases, viral diseases, and parasites in production systems for major aquaculture species, especially through early detection and prevention. Experts were invited to a special workshop in July 2001 to present information on biosecurity basics, to share their experiences implementing biosecurity practices in the poultry industry, shrimp farming, fish farming, and mollusk culture, to discuss potential air borne vectors of pathogens and transmission of pathogens through gamete exchange, and policy development to prevent and control the spread of disease. Detailed information reported at the workshop can be found in the proceedings, "Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables," published by the World Aquaculture Society in 2003 (Lee and O'Bryen 2003). This paper briefly highlights some of the major points and practices of biosecurity for various aquaculture production systems.

Biosecurity in poultry

Biosecurity is defined by the US poultry industry as "cumulative steps taken to keep disease from a farm and to prevent the transmission of disease within an infected farm to neighboring farms." (Hegngi 2003, p. 264). Biosecurity is a team effort, a shared responsibility, and an on-going process to be followed at all times. From the breeder to the hatchery, to grow out operators, biosecurity measures have to be observed to contribute to the success of the industry. The major components of biosecurity, as practiced by the poultry industry, include: isolation, traffic control, sanitation, and rodent and insect control. The purpose of these practices is to prevent the introduction of pathogens and to provide the best

living conditions for the health of the animals. In this way, the industry can minimize the risk of disease and insure the production of a clean food product. These principles can be applied to aquaculture practices in various ways to exclude the introduction of pathogens.

Biosecurity in aquaculture

Biosecurity in aquaculture has yet to be defined. It can mean different things to different stakeholders. Seafood consumers want to have an assurance that the product is safe to eat. Retailers have a responsibility to provide high quality seafood, and processors should follow Hazard Analysis and Critical Control Point (HACCP) guidelines to ensure that their products are safe for human consumption. At the farm site, workers need to know what practices decrease or increase the risk of a disease outbreak occurring. Investors seek to protect their investments from preventable losses. Indeed, the entire aquaculture industry is concerned about disease outbreaks. At the AIP Biosecurity workshop, practices at the industry level and policies in effect at the international and national level were discussed. Participants at the workshop defined biosecurity as "an essential group of tools for the prevention, control, and eradication of infectious disease and the preservation of human, animal, and environmental health." (O'Bryen and Lee 2003, p. 275).

The occurrence of disease is a combination of the health of the animal, the condition of the environment, and the presence of a pathogen. Klesius (2003) used the disease continuum model to illustrate how outbreaks of disease were the result of a weakened immune system of the culture animals, caused by neuroimmune changes resulting from stresses and infection. Therefore, excluding infectious agents and reducing stress are important in preventing disease outbreaks.

Biosecurity in shrimp farming

In general, biosecurity is more easily implemented in small, intensive, and controlled farming systems than in outdoor and large-scale operations (Horowitz and Horowitz 2003). Biosecurity measures in the shrimp industry can be seen as a two-pronged approach: excluding pathogens and eliminating pathogens when they are present.

Lightner (2003) discussed ways of excluding pathogens from stock (i.e., post larvae and broodstock), especially through the use of quarantine and specific pathogen-free (SPF) certified stocks, and restricting imports of live and frozen shrimp. Excluding vectors and external sources of contamination and preventing internal cross contamination were suggested methods for excluding pathogens from hatcheries and farms.

Horowitz and Horowitz (2003) described physical, chemical, and biological precautionary measures to be taken as well as a second line of defense against potential disease outbreaks. Physical measures are those that aim at preventing the intrusion of disease-carrying vectors to the farm site, and include physical barriers, water treatment, and quarantine. Chemical measures are those used to treat materials before they enter the facility. Chlorination and ozonization are often used to treat incoming water, and iodine and chlorine are used to treat other potential vectors such as tools, footwear, and clothing. Biological measures include the use of SPF shrimp, which are readily available commercially. A second

line of defense for the shrimp industry is to use specific pathogen-resistant shrimp, which, in addition to being disease-free, are resistant to specific diseases. Since shrimp do not develop a specific immune response, common immunostimulants, such as β-1-3 glucan, lipopolysaccharides, and peptidoglycans are used to improve the ability of the shrimp to prevent infection.

If a disease presents itself at a particular pond, effective biosecurity measures should prevent the complete loss of the crop and the spread of disease to other ponds. Lightner (2003) recommended an approach to eliminating pathogens at the stock level and partial disinfection at the facility level. To eliminate pathogens in post-larvae and broodstock, affected tanks and ponds should be depopulated, disinfected, and restocked with SPF shrimp. It may, however, be necessary to depopulate the entire stock and to fallow the entire facility if partial disinfection (using lime, chlorine, or drying) is not successful.

Horowitz and Horowitz (2003) suggested providing better environmental and biological conditions to the infected population to increase its ability to resist diseases. They discussed the following steps: a) effect physical measures (increase aeration, control temperature, improve the feeding regime, remove sludge and organic matter, and treat wastewater) to improve the environmental conditions, b) effect chemical measures, including control of PH and salinity, reduction of ammonia and nitrite, and application of antibiotics, and c) to use effective biological measures, consisting mainly of the use of probiotics containing a mix of bacterial species to establish beneficial microbial communities under culture conditions.

Biosecurity in finfish

Examples of biosecurity measures in finfish culture were presented by Yoshimizu (2003), Kent and Kieser (2003), and Breuil et al. (2003). Yoshimizu (2003) addressed biosecurity measures used in Japan against viral diseases in salmonids and flounder (*Paralichthys olivaceus*, *Verasper moseri*). These control strategies include both physical and biological aspects. The physical aspects start with cleaning and disinfecting measures in hatchery and production facilities. The next step is disinfecting incoming water and wastewater. Fish viruses, which are sensitive to either UV or total residual oxidants (TRO), are inactivated by a treatment of 10^4 to 10^5 μ sec/cm^2 UV or 0.1 to 0.5 mg/mL TROs for 1 min (Yoshimizu 2003). Ozonated seawater that contains TROs, however, is toxic to fish and should be removed with charcoal. For treating large volumes of wastewater, such as those from hatcheries, electrolysis is very effective (Yoshimizu 2003). Carefully regulating water temperatures to between 15 °C and 18°C has been shown to be effective at reducing Japanese flounder (*P. olivaceus*) rhabdovirus (HIRRV) infectivity (Yoshimizu 2003). Dedicated equipment, nets, brushes, etc., are disinfected with ozonated or electrolyzed seawater containing 0.5 mg/L of total residual oxidants (TROs) or chlorine for 30 min. In terms of the biological aspects of disease control, broodstock undergo health inspections to ensure they are pathogen-free, and the health of the fry is routinely monitored. Larvae that are cultured in disinfected water may need to have a normal intestinal flora restored. Larvae that are fed with bacteria isolated from the normal intestinal flora showed anti-infectious hematopoietic necrosis virus (IHNV) activity under challenges (Yoshimizu et al. 1992). Immunizing stocks,

using commercially available vaccines, is the most effective method for controlling salmonid diseases that cannot be excluded (Yoshimizu 2003).

In the mid-1980s, Atlantic salmon (*Salmo salar*) began being produced in British Columbia, and the industry now produces about 35,000 MT annually (Kent and Kieser 2003). The "eggs only" policy eliminates the introduction of many pathogens that require a live salmonid fish host. Any eggs that are imported into the area must have originated from certified specific disease-free sources, to ensure that diseases are not transmitted vertically. Kent and Kieser (2003) describe the methods that are used to disinfect Atlantic salmon eggs, which usually consists of 100 ppm iodine for 10 min. For species with eggs that require limited incubation time, eggs are disinfected with chlorine (0.6 mL 4-6% sodium hypochlorite/L) for 5 min. and then hatched in sterile water. Hatched larvae can be shipped under these conditions. Along with this policy, which also includes screening broodstock, disinfection, quarantine, and treatment of the effluent from quarantine facilities (5 ppm chlorine for 10 min and discharge to ground) are also included. As a result, nearly 23 million eggs have been safely imported into British Columbia since 1985.

In France, a pilot scale biosecure production system of sea bass (*Dicentrarchus labrax*) prevented vertical and horizontal transmission of nodavirus disease in broodstock to market size fish and avoided the use of antibiotics and anti-parasitic treatments, at a final production cost that was similar to traditional systems was presented by Breuil et al. (2003). Breuil et al. (2003) compared risk factors associated with the rearing of fish in various systems, and grouped them as meteorological events, such as storms and ocean swells, ecological events, such as plankton blooms and water pollution, pathological events, and other factors, such as mechanical problems. They concluded that recycling systems greatly reduce the risk of meteorological and ecological events except mechanical problems. By implementing biosecurity, the risk of pathological events can be reduced. The strategy combined the use of diagnostic tests for early detection and removal of nodavirus carriers to maintain healthy broodstock, control of specific bacterial populations in the recirculating system, i.e., use of a non axenic system, and treatment of wastewater with algae and reuse of the treated water (Breuil et al. 2003). Further mastering of the risks associated with rearing the fish in closed systems is possible. In general, treatment at the egg stage is expected to be the most effective.

Biosecurity in mollusc culture

Elston and War (2003) described the approach taken to biosecurity in mollusc culture in the US in terms of implementing health management and sanitation procedures for endemic diseases, and excluding non-endemic diseases. For endemic diseases, the health management procedures include assessing and understanding the state of health of individual and populations of cultured shellfish, early diagnosis of abnormal or pathological conditions, and preventing and correcting pathological conditions that may arise. Sanitation procedures are aimed at identifying and monitoring culture systems for contamination sources and management procedures to reduce or eliminate contamination. In intensive hatcheries and nurseries, pathogen-free algal stocks undergo surface sanitation in expanded culture and treated water is used with disease-free broodstock. Health assessments are critical at

metamorphosis of larval mollusc cultures, and sanitation and health management are two of the keys to ensure production of healthy juveniles (Elston and War 2003).

Non-endemic infectious diseases are excluded from mollusc culture operations usually through regulations set by authorities. Elston and War (2003) describe the elements of a highly effective regulatory biosecurity program for shellfish in Washington State. This program facilitates the transfer of established species within Washington State with minimal permitting requirements, evaluates the importation of species established in Washington State with a health history from the West Coast commerce region, and requires increasingly rigorous requirements for importations from non-established sources and from outside the West Coast region. Regulations also restrict imported shellfish from being released into state waters and can only be propagated in an approved quarantine facility.

Potential disease vectors

Two potential disease vectors are airborne pathogens and gametes. Bishop et al. (2003) showed that a fish protozoan pathogen (*Ichthyophthirius multifiliis*) was transmitted by an aerobiological pathway to infect fingerling channel catfish (*Ictalurus punctatus*) at a distance of 91 cm from the pathogen source tank. Biosecurity countermeasures that were suggested include covering tanks and aquaria, erecting barrier walls, lowering the humidity around culture tanks where possible, and rerouting air currents. Tiersch and Jenkins (2003) discussed biosecurity risks from cryopreservation of sperm from aquatic species, including transmission of viral, bacterial, fungal, and parasitic agents, and the introduction of exotic species. Numerous regulatory frameworks are in place at state, national, and international levels for controlling the transfer of cryopreserved materials. The US Department of Agriculture's plant germplasm system (USDA 2003) provides a model that may be adapted for use with cryopreserved gametes from aquatic animals. Tiersch and Jenkins (2003) propose a germplasm repository system for fish sperm, based on three physical locations and databases: a) an archival repository cryopreservation center for frozen samples, with quarantine facilities to evaluate quality, verify species, and screen for disease, b) a satellite repository with facilities for duplicative storage, and c) a working hatchery, where samples are thawed, fertilized, and monitored for disease. They also show how this system could be integrated into existing biosecurity systems, such as the one used to develop SPF lines of shrimp. Suggested biosecurity procedures include physical separation of animals and gametes if diagnostic microbiology is performed at the same facility, decontamination of media that comes into contact with gametes, and the establishment of protocols for disposal of contaminated samples and for collecting, processing, storing, and transporting samples. Proper labeling and good record keeping are essential for a successful biosecurity program (Tiersch and Jenkins 2003).

Aquaculture biosecurity policies

Aquaculture biosecurity policies vary from farm-level to the international level, and between areas at each of these levels, but several characteristics are essential if aquaculture biosecurity polices are to be successfully implemented (Scarfe 2003). These common

characteristics include: a) science-based decision making, b) economical and sociopolitical rationales, c) standardized and uniform methods, d) relative ease of application, e) wide recognition, f) vertical and horizontal integration, application, and agreement, g) consistent enforcement, and h) a primary focus on prevention, but with contingencies in place for control and management, or eradication.

A few of the major instruments for dealing with biosecurity at the global level are the World Trade Organization's Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement), the Food and Agriculture Organization of the United Nation's Codex Alimentarius and Codes of Conduct, and the International Council for the Exploration of the Sea's (CICES) Code of Practice on Introductions and Transfer of Marine Organisms (see Scarfe 2003, Table 1). The primary focus is on enhancing or protecting trade through biosecurity.

Issues regarding aquatic animal health are usually deferred to the Office International des Epizootices (OIE) (Scarfe 2003). Its mission is to inform governments of the occurrence and course of diseases throughout the world and of ways to control these diseases, to coordinate studies devoted to the surveillance and control of animal disease, and to harmonize regulations for trade in animals and animal products among its 158 member countries (D. Lightner, personal communication, July 2001). The *OIE International Aquatic Animal Health Code* and accompanying *OIE Diagnostic Manual for Aquatic Animal Diseases* (OIE Code and OIE Manual, respectively) are accepted by the member countries as international guides to preventing the movement of aquatic animal pathogens and diseases (Scarfe 2003). Key elements of the OIE Code in terms of biosecurity of aquatic animals include the General Provisions, the lists of diseases, and the section on Health Control and Hygiene. The General Provisions include general definitions, a section on Import Risk Analysis, and Import/Export Procedures. Lists of diseases of finfish, molluscs, and crustaceans are prioritized according to their significance because of their potential rapid spread, serious public health consequence, or importance in trade. The Health Control and Hygiene section includes procedures for disinfection of fish farms, mollusc farms, crustacean farms, and of fish eggs with iodine (Scarfe 2003).

At the national level, Australia has a comprehensive biosecurity program (AQUAPLAN) in place that provides an overall management strategy for aquatic animal health (Findlay 2003). This program applies integrated management strategies from the borders to individual farms or specific areas, with international linkages to OIE guidelines that have helped Australia to gain a trustworthy trade reputation. The Australian Quarantine and Inspection Service and Biosecurity Australia manage AQUAPLAN's quarantine program. Biosecurity Australia has an *Import Risk Analysis Handbook* (AQIS 1998) that details the process of import risk analysis, which is pivotal to every program within AQUAPLAN. The components, which are outlined in the SPS Agreement, generally involve a combination of qualitative or semi-quantitative risks and likelihoods of a disease incursion and its qualitative consequences (Findlay 2003). In descriptive terms, AQUAPLAN is a very conservative approach to quarantine risk, i.e., a very low acceptable risk for imported aquatic animals. Its success can be measured in improved aquatic animal health management in Australia, increased productivity and improved sustainability of its aquaculture, improved market access, and better protection for Australian aquatic ecosystems (Findlay 2003).

Conclusion

Biosecurity can be applied to aquaculture production systems through a variety of management strategies and by following internationally agreed upon policies and guidelines. In addition, there are a variety of risk assessments that can be used for aquatic animal diseases of finfish, molluscs, and crustaceans (Lee and Bullis 2003). The key elements of biosecurity can be summarized as reliable sources of stock, adequate diagnostic and detection methods for excludable diseases, disinfection and pathogen eradication methods, best management practices, and practical and acceptable legislation. Nevertheless, it is almost impossible to determine the economic benefits of a biosecurity program if there is no disease outbreak, and aquaculture producers may be reluctant to adopt biosecurity measures that appear to be an additional cost. A disease outbreak in one area, however, in addition to its economic consequences in that area, may cause unintended consequences in other parts of the world. The adage, "think globally and act locally" should apply to aquaculture production in the 21st century, as international standards for diagnosing and reporting diseases are adopted, methods for excluding diseases from culture systems are made integral to culture operations, and acceptable methodology for treating them is established.

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The Impact of Diseases on Crab and Lobster Fisheries

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Abstract:

Diseases are an integral part of crustacean fisheries. As with most fisheries, the question arises as to why one should be concerned about diseases when nothing can be done to limit their effects on the fished population. First, natural mortality is often assumed to be 0.2 in pre-recruits in many fishery models. Unfortunately, background levels can be much higher, especially during outbreaks of pathogens such as *Hematodinium perezi* or *Paramoeba perniciosa* where mortalities to the pre-recruit populations can approach 100% in endemic locations. Stock assessments and fishery models must incorporate losses to diseases if they are to be used in managing the resource. Second, several parasitic diseases cause marketability issues through stunting of the host or by causing unsightly lesions in the meat. Poor marketability negatively influences public opinion about quality of product. Third, certain fishing practices such as holding animals in receivers or impoundments and transporting animals between watersheds may help to spread diseases. By understanding transmission and pathogenicity of a disease, one can curtail or minimize such practices. Fourth, with the advent of live shipping of crabs and lobsters, there is an increased potential for the inadvertent introduction of pathogenic agents to new regions. This is not a trivial issue as introduced diseases have damaged the shrimp industry worldwide and have marginalized the aquaculture of abalone in California.

Diseases of the crabs and lobsters affect fecundity, recruitment, and mortality; yet, there are few practical responses to control or mitigate impacts of diseases in crustacean fisheries. Simple measures such as "culling" infected individuals on station or within a watershed, culling or removing dead animals to onshore fertilizer processing plants, limiting transportation of live animals, and changing "baiting practices" may limit the spread of pathogens to new locations. Changes in fishing policies may also be warranted. Regulations on minimum size may enhance populations of parasites that stunt their hosts, and the accumulation of stunted crabs may further impact the fishery. By using outreach or other education programs, fishermen could practice destroying stunted and parasitically castrated crabs, but many fishermen are loathe to keep or kill small crabs for fear of penalties or infractions from enforcement agencies. Further, many state or regional agencies have monitoring programs for stock assessments. Rhizocephalan barnacles, which cause alterations and appear similar to egg masses, could easily be included in such monitoring protocols. The wealth of information from such programs would enhance our understanding of disease prevalence and association with host and environmental variables and help to document impacts on the individual hosts and the fished populations.

Crustacean Hyperglycemic Hormone and Hemolymph Metabolites: Stress Responses in Two Lobster Species

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Abstract

Our laboratory has developed several methods for the quantification of stress in crustaceans. An ELISA was developed for the crustacean hyperglycemic hormone (CHH) from the American lobster (*Homarus americanus*). It is sensitive to as little as 0.2 fmol of peptide. Increases in hemolymph CHH were observed under conditions of hypoxia, elevated temperature, and altered salinity. In addition, elevated CHH concentrations were observed in Norway lobsters (*Nephrops norvegicus*) that were parasitized with the dinoflagellate *Hematodinium* and in other lobsters following the stress of capture by trawling.

Introduction

Stress responses occur in all animals when regulated physiological systems are extended beyond their normal range by external stressors. Failure of all or part of the integrated homeostatic response may lead to increasing physiological disturbance and ultimately death. Indicators of such stress responses are therefore useful in assessing the short-term well-being or long-term health status of an animal and such indicators have received considerable attention in commercially important decapod crustacean species (Paterson and Spanoghe, 1997).

Hyperglycemia as a response to various kinds of stress is well documented in decapod crustaceans. Regulation of hemolymph glucose is mediated by the release of the crustacean hyperglycemic hormone (CHH) that is synthesized in the eyestalk x-organ and stored prior to release from the sinus gland (for review see Böcking *et al.*, 2002). We have developed an enzyme-linked immunosorbent assay (ELISA) for CHH as a tool for the quantification of various environmental stresses in lobsters (hypoxia, thermal stress, salinity

stress). We also examined the effects of the stresses imposed by parasitism and capture upon CHH and hemolymph metabolites.

Materials and Methods

Animal sampling

Sampling of hemolymph and methods for inducing stress for CHH and metabolite measurements in American lobsters (*Homarus americanus*) have been described (Chang *et al.*, 1998). Norway lobsters (*Nephrops norvegicus*) were captured from two sites on the west coast of Sweden. One subset of lobsters (n=20) was captured using a standard otter trawl, towed for 1 h in the Skagerrak Sea area at the mouth of the Gullmarsfjord. Another subset (n=20) was captured using baited creels set within the same area (57°39.927N, 11°29.658E). Trawled and creeled lobsters were sampled immediately upon landing. One ml of hemolymph was drawn from the base of the 5th pereiopod of each lobster into a 2-ml syringe fitted with a 23-gauge needle and frozen immediately by immersing the syringe into liquid nitrogen. Samples of hepatopancreas were removed, placed into vials and frozen as above. Upon reaching the laboratory, all samples were lyophilized overnight in a freeze dryer prior to storage at 4°C. Characterization of the parasitization of *N. norvegicus* by the dinoflagellate *Hematodinium* sp. has been published (see Field and Appleton, 1995).

CHH analysis of hemolymph

Production of an antibody against purified CHH-A from H. americanus and development of the ELISA have previously been described (Chang et al., 1998). Prior studies demonstrated that N. norvegicus CHH could be detected using this antibody (Stentiford et al., 2001). In the current study, freeze-dried plasma samples were resuspended to their original volume with water and assayed for CHH with ELISA. Due to the lack of available purified N. norvegicus CHH, it was not possible to quantify absolute concentrations of CHH in N. norvegicus. The data are therefore presented as Orconectes limosus equivalents, which were used as the standards and which allow relative quantification in N. norvegicus.

Glycogen analysis of hepatopancreas

For determination of glycogen, 400 µl of 30% potassium hydroxide were added to 20 mg of the freeze-dried samples of *N. norvegicus* hepatopancreas. The samples were heated for 20 min in a water bath maintained at 95-100°C. Samples were cooled and added to 700 µl absolute ethanol before being placed on ice for 2 h. Following precipitation, samples were spun at 17,000*g* for 10 min and the supernatant discarded. One ml of water was added to each sample before sonication. Fifty µl of each sonicated sample were incubated at 95-100°C in 1 ml of anthrone reagent before measurement of total glycogen (see Carroll *et al.*, 1956).

Glucose analysis of hemolymph

Glucose concentration in the lobster hemolymph was measured using the glucose oxidase method (Boehringer-Mannheim) in a multi-well plate method as described by Webster (1996). Briefly, 50 µl samples of deproteinized plasma were added to 450 µl of 0.2 M phosphate buffer (pH 7.4) and 100 µl samples of this solution were used in the assay with 200 µl of the enzyme chromogen reagent. Concentrations of plasma glucose were read from a standard curve constructed for glucose.

Lactate analysis of hemolymph

Lactate was measured using a kit (Sigma #826). The reagents were prepared according to its protocol. Anticlotting buffer (glycine ethyl ester; Chang *et al.*, 1998) was added to hemolymph (1:1, v:v) and centrifuged. Sample supernatants (20 µl) and standards (10 µl and 10 µl of anticlotting buffer) were added to individual wells of a 96-well plate. Reagent (lactate dehydrogenase and nicotinamide adenine dinucleotide) (200 µl) was added to each well. After mixing and a 10-min incubation, the plate was read at 540 nm. Concentrations of lactate were determined from the standard curve.

Results

CHH in H. americanus

We observed that emersion is a potent stimulator for the elevation of hemolymph CHH (Chang *et al.*, 1998). **Figure 1** shows that it increases from resting values of 4.0 fmol/ml to 168.1 fmol/ml after 4 h emersion. Although handling stress slightly increases CHH, the additional stress of emersion is significantly above the handling stress observed in immersed controls (from 11.3 to 20.6 fmol/ml) held at the same temperature (13°C) as the emersed lobsters.

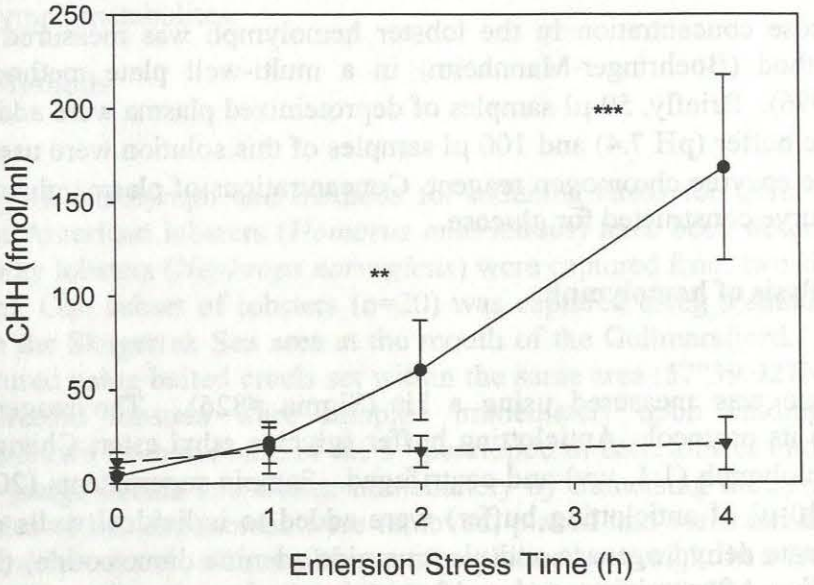

Figure 1. Effects of emersion on *H. americanus* hemolymph CHH (circles, solid line). Lobsters (n=7) were sampled as previously described (Chang *et al.*, 1998). Means±SD are shown. Control data are represented by the triangles with the broken line (n=8). Asterisks indicate significant differences from immersed controls at $P<0.01$ (**) and at $P<0.001$ (***). Modified from Chang *et al.* (1998).

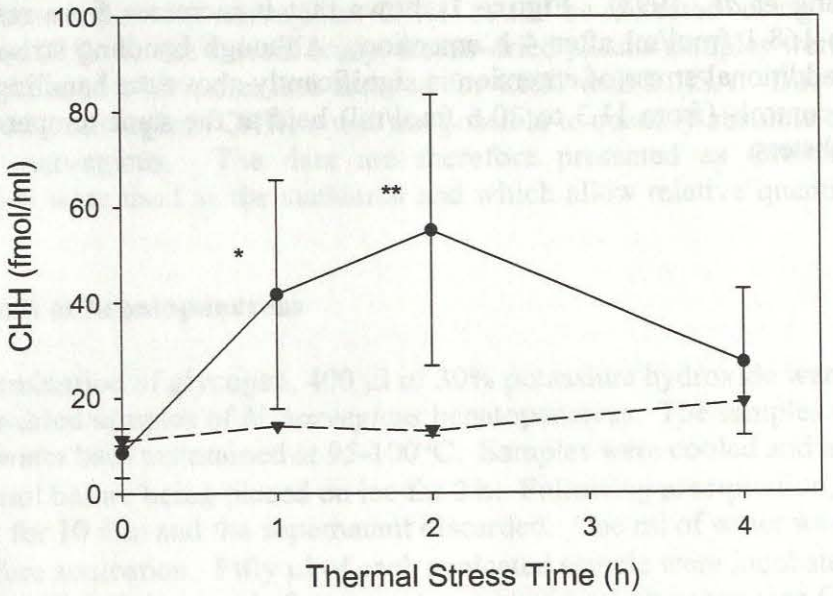

Figure 2. Hemolymph CHH concentrations (means±SD; circles, solid lines) in immersed *H. americanus* held at 23°C (n=8). Hemolymph was sampled and assayed by ELISA. Controls (triangles with broken line) are the same animals used in Fig. 1 (error bars have been

omitted). Asterisks indicate significant differences from 13°C controls at $P<0.05$ (*) and at $P<0.01$ (**). Modified from Chang *et al.* (1998).

Thermal stress caused an increase in hemolymph CHH. **Figure 2** shows that a 10°C change in temperature to 23°C caused an increase in CHH relative to ambient (13°C) controls. No significant changes in hemolymph CHH were observed following a 5° temperature elevation nor were changes seen during cold stress (data not shown). Both hyposalinity (50%) and hypersalinity (150% seawater) resulted in significant alterations in hemolymph CHH after 2 h. This elevation in CHH relative to the controls was not significant at later time points **(Fig. 3)**.

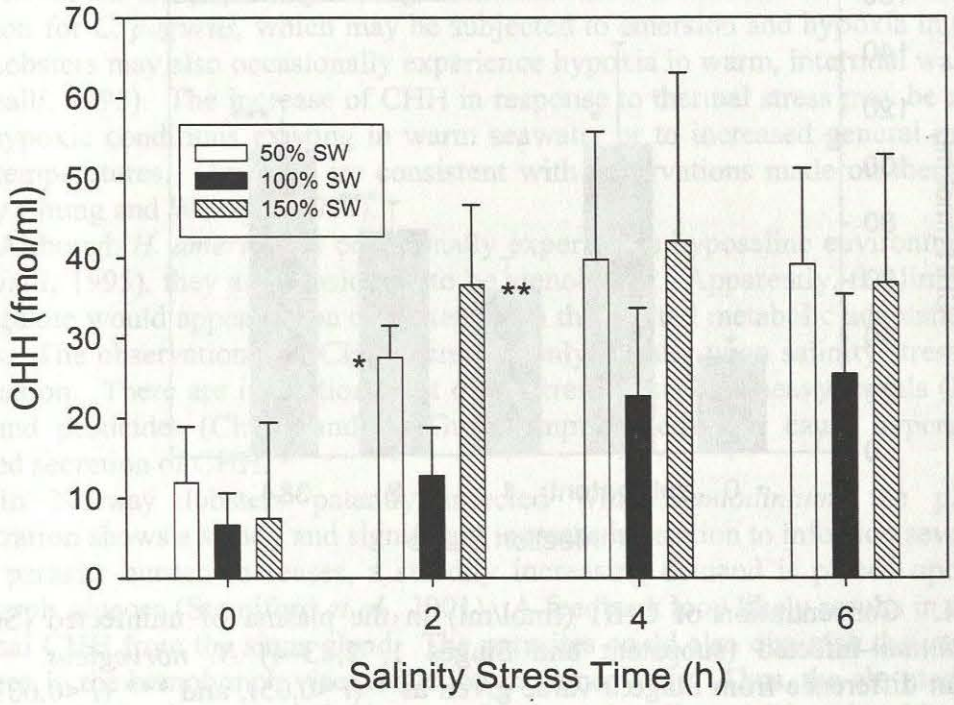

Figure 3. Hemolymph CHH concentrations (means±SD) in immersed *H. americanus* held in 50% (white bars), 100% (black bars), or 150% (hatched bars) seawater (n=5 for each salinity). Hemolymph was sampled and assayed by ELISA. Asterisks indicate significant differences from the 100% seawater controls at $P<0.05$ (*) and at $P<0.01$ (**). Modified from Chang *et al.* (1998).

CHH and hemolymph metabolites in N. norvegicus

The mean concentration of CHH in the plasma of uninfected *N. norvegicus* was 32.2 fmol/ml. The mean concentration of CHH in the plasma of lobsters subpatently infected with the dinoflagellate *Hematodinium* sp. (107.65 fmol/ml) was significantly higher than that of Stage 0 (uninfected) animals ($P<0.05$). At Stage 1 (light patent infection), the mean concentration was higher (though not significantly, $P=0.057$) than that of Stage 0 and lower (though not significantly, $P=0.070$) than that of subpatently infected lobsters. In later stages

of patent *Hematodinium* infection, the plasma CHH concentration was significantly higher than that of uninfected animals [Stage 2 (77.2 fmol/ml, $P<0.001$) and Stage 3–4 (106.6 fmol/ml, $P<0.001$)]. The significant increase in plasma CHH concentration between Stage 1 and Stage 3–4 animals ($P<0.05$) and the almost significant increase between Stage 1 and Stage 2 animals ($P=0.080$) suggest that the titer of CHH increases with the severity of patent infection, but also that initiation of subpatent infection may involve a temporary rise in plasma CHH titer. It is interesting to note, however, that the mean plasma CHH titer of subpatently infected lobsters is not significantly different from that of Stage 3–4 lobsters ($P=0.997$) **(Fig. 4).**

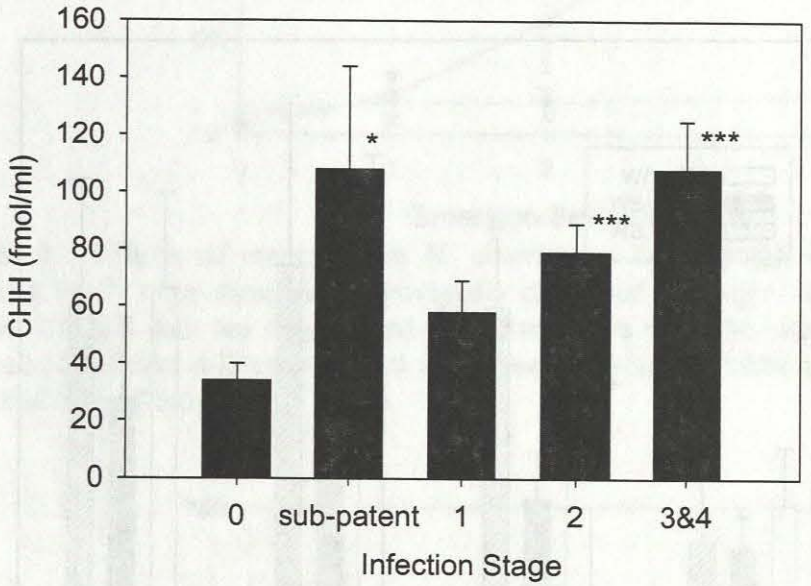

Figure 4. Concentration of CHH (fmol/ml) in the plasma of uninfected (Stage 0) and *Hematodinium*-infected (subpatent and Stages 1, 2, 3–4) *N. norvegicus*. Statistically significant difference from Stage 0 value given as * ($P<0.05$), and *** ($P<0.001$). Stage 0, n=22; subpatent, n=8; Stage 1, n=22; Stage 2, n=33; Stage 3–4, n=15. Modified from Stentiford *et al.* (2001).

The mean concentration of CHH in the hemolymph of lobsters captured by trawling was 97.7 fmol/ml and was significantly higher than in those lobsters captured by passive trapping (2.34 fmol/ml) (Mann Whitney test, W=55.0, $P<0.001$). The mean concentration of glycogen in the hepatopancreas of lobsters captured by creeling (8.59±1.4 µg/g dry weight) was lower than those captured by trawling (12.26±2.41 µg/g dry weight) but the difference was not significant (Mann-Whitney test, W=220.0, $P>0.05$).

Glucose concentration in the hemolymph of lobsters captured by trawling was 197.5±28.6 µg/ml. The mean concentration of glucose in the hemolymph of lobsters captured by creeling (138.3±12.8 µg/ml) was lower, but the difference was not significant (ANOVA test, F=5.06, P>0.05). The concentration of lactate in the hemolymph of lobsters captured by trawling was 607.1±14.8 mg/ml. The concentration of lactate in the hemolymph of lobsters captured by

creeling (12.9±5.0 mg/ml) was significantly lower than in those captured by trawling (ANOVA test, $F=55.0$, $P<0.001$).

Discussion

We have demonstrated that various environmental stresses and physiological states resulted in elevations in the hemolymph CHH concentration in American lobsters. These stresses included emersion (producing hypoxia), temperature elevation, and salinity changes. Our results are in agreement with those of Webster (1996) on *Cancer pagurus*. He found that emersion causes a significant increase of CHH in the hemolymph 15 min after emersion. Similarly, in our lobsters, a significant increase was measurable after 20 min. Webster (1996) discussed the physiological significance of this mechanism of endocrine metabolic adaptation for *C. pagurus,* which may be subjected to emersion and hypoxia in the intertidal zone. Lobsters may also occasionally experience hypoxia in warm, intertidal waters (Lawton and Lavalli, 1995). The increase of CHH in response to thermal stress may be related either to the hypoxic conditions existing in warm seawater or to increased general metabolism at higher temperatures. Our data are consistent with observations made on thermal stress on crabs by Chung and Webster (1996).

Although *H. americanus* occasionally experiences hyposaline environments (Lawton and Lavalli, 1995), they are considered to be stenohaline. Apparently, the limited ability to osmoregulate would appear to be consistent with the limited metabolic adaptation to salinity changes. The observation that CHH increases only slightly upon salinity stress may reflect this situation. There are indications that other stresses, such as heavy metals (Reddy *et al.,* 1996) and pesticides (Chang and De Guise, unpublished) may cause hyperglycemia via increased secretion of CHH.

In Norway lobsters patently infected with *Hemtodinium*, the plasma CHH concentration shows a steady and significant increase in relation to infection severity (Fig. 4). As the parasite burden increases, a steadily increasing demand is placed upon the hosts' hemolymph glucose (Stentiford *et al.*, 2001). A feedback loop likely results in the release of additional CHH from the sinus gland. The parasites could also diminish the partial pressure of oxygen in the hemolymph via a reduction in hemocyanin. Thus, the elevated hemolymph CHH concentration in patent infection may be due primarily to a "functional hypoxia" in the infected lobster, which elicits a cascade response similar to that seen during the "environmental hypoxia" caused by emersion.

Our results suggest that a one-hour trawling duration causes a small but not statistically significant increase in the hemolymph glucose of Norway lobsters. The hepatopancreas of lobsters captured by trawling had a slightly increased (though not significant) concentration of glycogen. The most significant differences between trawled and creeled lobsters were observed in the concentrations of lactate and CHH in the hemolymph. Lactate was elevated almost 40-fold and CHH was elevated almost 50-fold in the hemolymph of trawled lobsters. Reduced glucose normally results in increased CHH release from the sinus gland via a negative feedback loop. Since glucose was not reduced in the hemolymph of trawled animals, it is unlikely that the elevated CHH is caused by this mechanism. Instead, the elevated hemolymph lactate, a feature known to drive the positive feedback of CHH release from the sinus gland, is more likely to lead to the elevated CHH concentration observed in the trawled lobsters.

Since trawled lobsters undergo exhaustive exercise in an attempt to escape capture by a typical trawl net and may further undergo additional exercise within the cod-end of the net, it is likely that the elevated hemolymph lactate was derived from the large muscles responsible for flexion of the abdomen. Excess lactate is transferred from the muscle to the hemolymph in an attempt to reduce tissue acidosis. With continued exercise it accumulates in the hemolymph.

We believe that the results presented in this paper demonstrate that measurements of hemolymph CHH (and selected circulating metabolites) will be useful for monitoring a variety of stress responses in lobsters and to study the role of CHH in the metabolic regulation of crustaceans. Our CHH research will complement other stress indicators, such as the induction of stress proteins (Spees *et al.*, 2002a,b, 2003). These studies should have utility in the monitoring of ecosystem health and in improving fisheries and aquaculture practices.

Acknowledgments

We thank W.A. Hertz for assistance with animal care. This work was funded in part by a grant from the National Sea Grant College System, National Oceanic and Atmospheric Administration (NOAA), US Department of Commerce, through the California (Project R/A-111A) and Connecticut (Project LR/LR-1) Sea Grant College Programs (to ESC). The work on Norway lobsters was funded in part by the UK Ministry of Agriculture, Fisheries and Food (Grant No. MAFF MF0221) and by a European Union LSF grant held at the KMRS Marine Station, Sweden (both to DMN). The views expressed in this study are those of the authors and do not necessarily reflect the views of NOAA or any of its subagencies. The US Government is authorized to reproduce and distribute for governmental purposes. Contribution Number 2193, Bodega Marine Laboratory, University of California at Davis.

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Feeding Ecology of an Abalone, Haliotis discus hannai, in Their Early Life Stages

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Key Word: abalone, feeding, growth, *Haliotis discus hannai* nutrition, survival

Abstract

Recent studies on the feeding ecology of abalone, *Haliotis discus hannai*, during the early life stages were reviewed in this article. The feeding habits of *H. discus hannai* changed with growth, and three major changes in the feeding were identified. The first change occurred at the time of the metamorphosis of 0.28 mm in shell length (SL), namely a shift from lecithotrophy (yolk absorption) to exogenous feeding. The energy source of abalone is gradually transferred from yolk to particulate food after metamorphosis by the size of 0.3-0.4 mm SL. Several days of food limitation after metamorphosis leads to a failure to shift to exogenous feeding. In the second change, when the SL reached values of about 0.6-0.8 mm, post-larvae were able to digest diatom diet. They grew more rapidly, feeding on efficiently digested diatoms, whereas those fed poor diatom diet will generally grow slowly. The differences in the dietary value of diatoms for abalone were controlled mainly by the digestibility of diatoms. The final change consisted of a shift from a biofilm-dominated diet to a macroalgal dominated diet. Abalone (>1.8 mm SL) were able to utilize juvenile macro algae efficiently. Juvenile macroalgae provide a much higher biomass per unit area than small-volume, two dimensional diatom films. The energetics of diatom ingestion became insufficient to support rapid growth. These changes in feeding habits were closely related to ontogenetic changes in the digestive enzyme activities and the development of the radula morphology. The activity of macroalgal polysaccharide-degrading enzymes showed marked increase at 2 mm SL. The morphological development in the radula occurred mostly in abalone less than 4 mm SL, to complete adult radula which is suitable to graze macroalgae.

Introduction

The food deprivation tolerance of newly metamorphosed *H. discus hannai* is extremely low, and limiting food over several days has a harmful effect on the survival of post-larvae (Takami *et al.*, 2000). Growth rates in the early life stages of *H. discus hannai* are considerably affected by the diet and the ability of the individuals to utilize available food (Kawamura and Takami, 1995; Kawamura *et al.*, 1995; Seki, 1997; Takami *et al.*, 1997a, b). In many abalone hatcheries, low survival and growth rates are often observed in the first few months, and these are probably caused by inadequate or inappropriate diets. Thus, understanding of the abolone feeding ecology in the early life stages is considered important for the improvement of rearing techniques in abalone hatcheries.

This article will review studies on the feeding ecology in the early life stages of *H. discus hannai* with a focus on the feeding transitions associated with the development of the digestive ability.

Feeding Transitions in Abalone *Haliotis discus hannai*

Shift from Lecithotrophy to Particulate Matter Feeding

Abalone species have planktonic larval stages before metamorphosing to benthic stages. Larval abalone are lecithotrophic (non-feeding) and carry a yolk derived from the egg that fuels larval life and metamorphosis. However, this diet could be supplemented by dissolved organic matters (Manahan and Jaeckle, 1992; Shilling *et al.*, 1996). Larval *H. discus hannai* require about 3-4 days at 20°C before becoming able to metamorphose (Seki and Kan-no, 1977). When settlement cues are provided, able larvae attach to a substratum and subsequently metamorphose into benthic post-larvae.

Larval abalone delay metamorphosis if they fail to detect an appropriate environmental stimulus (Roberrts and Lapworth, 2001; Takami *et al.*, 2002). Delayed metamorphosis of lecithotrophic larvae causes a depletion of yolk reserves, which has a negative effect on metamorphosis success, post-metamorphic survival, and growth. Larval *H. discus hannai* remained competent to metamorphose in extended larval period and can metamorphose successfully after 19 days from fertilization at 20°C. Survival and growth of the post-larvae are influenced by an extended larval swimming period. Post-larval survival and growth rates do not differ significantly between larval swimming periods ≤ 15 days, but become significantly lower for post-larvae with a 19-day swimming period, compared with post-larvae with a 15-day swimming period. These results suggest that the amount of residual yolk reserves available for post-larvae declines as the duration of their larval period become longer and has a significant effect on the survival and growth of newly metamorphosed individuals as an initial energy source (Takami *et al.*, 2002).

During the metamorphosis, larval abalone shed the velum, develop enlarged gills and a foot, and start peristomal shell formation. Post-larval abalone commence particle feeding using the radula immediately after completion of the metamorphosis. Newly metamorphosed post-larvae still have a visible yolk and initial survival and growth can still be supported by the yolk supply in addition to particle feeding when the shell length (SL) increases from 0.28 mm at the time of metamorphosis to 0.5 mm. The primary nutrition source for post-larval *H. discus hannai* is gradually transferred from yolk to particulate food after the metamorphosis (Takami *et al.*, 2000).

Under hatchery conditions, *H. discus discus* show massive mortality when the SL is 0.5 mm if there is a lack of food (Ohashi, 1993). Under experimental conditions, *H. discus hannai* with a SL below 0.5 mm die when they are fed unsuitable food sources (Takami *et al.*, 1997a, b, 2000). In the natural habitat, a number of dead or dying *H. discus hannai* with a SL of 0.4-0.5 mm were often observed, presumably due to starvation (Sasaki and Shepherd 2001). It was suggested that food limitation for several days after metamorphosis led to a failure to shift to exogenous feeding (Takami *et al.*, 2000). Thus the initial habitat should provide enough food for the post-larval abalone.

In natural habitats, larval *H. discus hannai* preferentially settle on crustose coralline algae (CCA) and grow on CCA for at least several months (Saito, 1981; Sasaki and

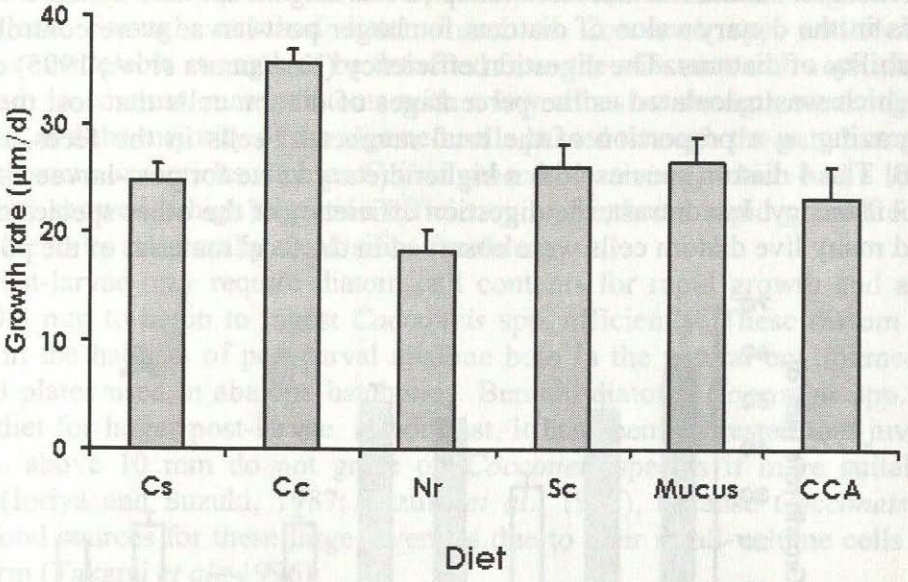
Shepherd, 1995, 2001; Takami, 2002). Grazing gastropods are usually found at high densities on CCA (Ayling, 1981; Choat, 1982; Kawamura *et al.* 1992; Takami, 2002), and CCA rely on their grazing to prevent surfaces from being covered with competitively superior algae (Paine, 1992; Steneck, 1983). Grazing-resistant algae with strongly adhesive prostrate forms such as benthic diatoms *Cocconeis* spp. tend to dominate on the CCA surface, and dense patches of *Cocconeis* spp. were often observed on CCA where post-larval and juvenile abalone were present (Kawamura *et al.* 1992; Takami, 2002).

In many Japanese abalone hatcheries, pre-grazed plates are used as substrata for the settlement of larvae and as rearing plates for post-larvae. The pre-grazed plates are first covered with a film of naturally occurring microalgae, and then the plates are grazed by juvenile or adult abalone (> 10 mm SL). Grazing-resistant algae such as *Cocconeis* spp. also dominate on the plates (Ioriya and Suzuki, 1987; Suzuki *et al.*, 1987). Both CCA and pre-grazed plates strongly induce larval settlement and are considered to supply adequate food sources for post-larval abalone.

In post-larval habitats in the natural environment and hatcheries, (1) CCA themselves, (2) benthic diatoms which dominate on CCA and on pre-grazed plates such as *Cocconeis* spp., and (3) the trail mucus left by herbivorous gastropods, including juvenile or adult abalone creeping on the substrata, appear to be food sources for the newly metamorphosed abalone. The growth rates of the newly metamorphosed *H. discus hannai* (< 0.6-0.8 mm SL) were compared among mono-cultured diatom diets including *Cocconeis* spp., CCA *Lithophyllum yessoense* which was not attached to any diatoms, and the trail mucus of juvenile *H. discus hannai* (30 mm SL) to determine the possible food sources for post-larvae on CCA and pre-grazed plates (Kawamura and Takami, 1995; Takami et al. 1997a, b).

Differences in the diatom species feed did not alter appreciably the growth rates of the post-larvae (**Fig. 1**). Diatom species *Navicula ramosissima* and *Stauroneis constricta* passed through the post-larval abalone gut alive, whereas *Cylindrotheca closterium* were ruptured and lost their cell contents. Post-larvae fed *Cocconeis scutellum* did not ingest any diatom cell material, probably due to the high adhesive strength of this species. These results and observations suggest that smaller post-larvae (< 0.6-0.8 mm SL) can grow well without high levels of absorption of the diatom cell contents. It appears that the extracellular substances of diatoms are an important source of food for smaller post-larvae (Kawamura and Takami, 1995).

The newly metamorphosed individuals (< 0.5 mm) fed CCA showed comparable growth rates to those of individuals fed on benthic diatoms (**Fig. 1**). Since post-larvae do not ingest CCA fragments, individuals utilize biofilm components such as extracellular products plus bacteria (Takami *et al.*, 1997a). The trail mucus of conspecific juveniles also supports adequate growth of post-larvae smaller than 0.7 mm (**Fig. 1**). On the pre-grazed plates, the extracellular materials of *Cocconeis* spp. and conspecific trail mucus were possibly the main food sources for smaller post-larvae (Takami *et al.*, 1997b).



TAKAMI FIG. 1

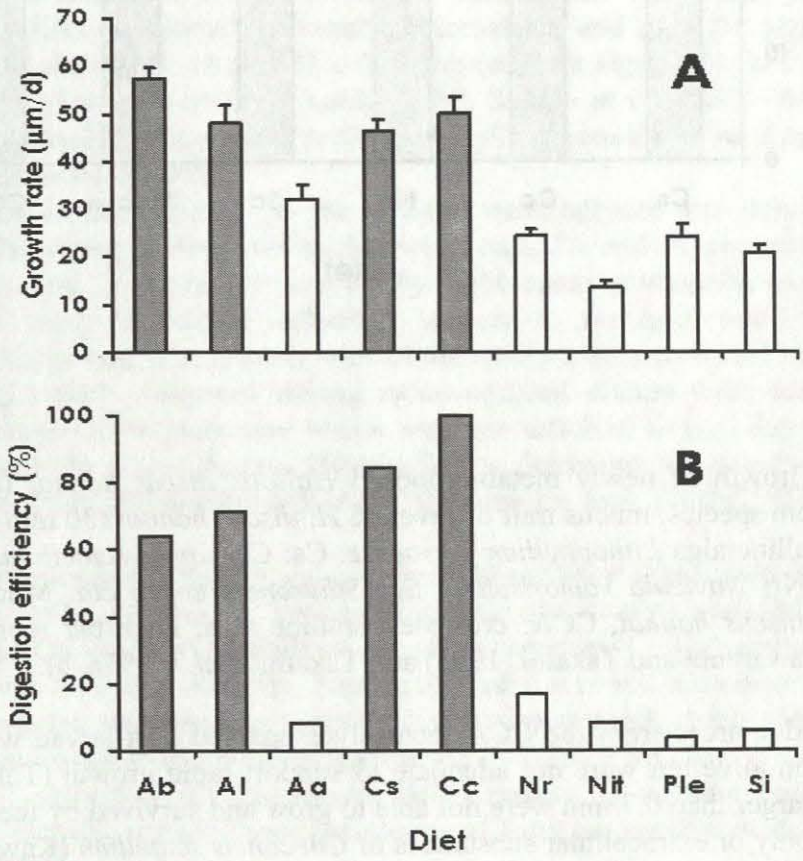
Figure 1: Growth of newly metamorphosed *Haliotis discus hannai* (µm per day) fed 4 benthic diatom species, mucus trail of juvenile *H. discus hannai* (30 mm in shell length) and crustose coralline alga *Lithophyllum yessoense*. Cs: *Cocconeis scutellum*, Cc: *Cylindrotheca closterium*, Nr: *Navicula ramosissima*, Sc: *Stauroneis constricta*, Mucus: mucus trail of juvenile *H. discus hannai*, CCA: crustose coralline alga. Each bar represents mean + SE. Data from Kawamura and Takami (1995) and Takami *et al.* (1997a, b).

Food sources from the CCA themselves enabled post-larvae with a SL above 0.5 mm to remain alive but were not adequate to support rapid growth (Takami *et al.*, 1997a). Post-larvae larger than 0.7 mm were not able to grow and survived by feeding on conspecific trail mucus only or extracellular substances of *Cocconeis scutellum* (Kawamura and Takami, 1995; Takami *et al.*, 1997b). Larger post-larvae need to utilize diatom cell contents for adequate growth.

Changes in Digestibility of Diatoms

Post-larval abalone with a SL above 0.8 mm became able to digest diatom diets and grew more rapidly on efficiently digested diatom species. The term "digestibility" refers to the proportion of diatom cells that lost cell contents when ingested and passed through the abalone gut (Kawamura *et al.*, 1995). **Figure 2A** shows the growth rates of larger post-larvae (1-2 mm) fed 9 diatom species. All the post-larvae fed these algae displayed an active feeding behavior but significant differences were observed between the growth rates of abalone fed different diatom species. The mean growth rates of the post-larvae fed the diatom species, *Achnanthes brevipes*, *A. longipes*, *Cocconeis scutellum*, and *Cylindrotheca closterium*, were significantly higher than those of the post-larvae fed the species, *Amphora*

angusta, *Navicula ramosissima*, *Nitzschia* sp., *Pleurosigma* sp., and *Synedra investiens*. The differences in the dietary value of diatoms for larger post-larvae were controlled mainly by the digestibility of diatoms. The digestion efficiency (Kawamura *et al.*, 1995) of each diatom species, which was calculated as the percentages of diatom cells that lost their contents by abalone grazing as a proportion of the total number of cells in the feces, is presented in **Figure 2B**. The 4 diatom species with a higher dietary value for post-larvae showed a higher digestion efficiency. In contrast, the digestion efficiency of the other species of diatoms was lower, and many live diatom cells were observed in the fecal material of the post-larvae.



TAKAMI FIG. 2

Figure 2. The mean (+SE) growth rates of post-larval *Haliotis discus hannai* with a shell length of 1-2 mm (µm per day) fed 9 benthic diatom species (A) and the mean digestion efficiency of diatoms grazed by post-larval *H. discus hannai* (B). Ab: *Achnanthes brevipes*, Al: *Achnanthes longipes*, Aa: *Amphora angusta*, Cs: *Cocconeis scutellum*, Cc: *Cylindrotheca closterium*, Nr: *Navicula ramosissima*, Nit: *Nitzschia* sp., Ple: *Pleurosigma* sp., Si: *Synedra investiens*. Data from Kawamura *et al.* (1995).

A limited number of diatoms showed high digestion efficiencies and induced rapid growth in larger post-larvae (Kawamura *et al.*, 1995, 1998; Roberts *et al.*, 1999a). Diatom

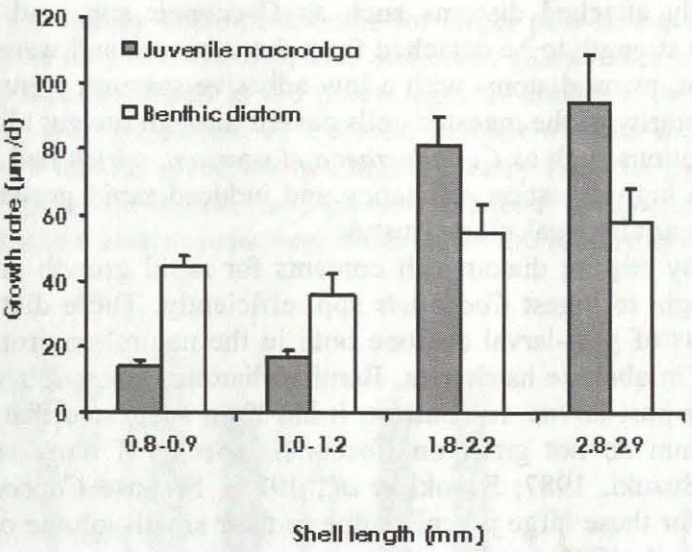
attachment strength was one of the factors that affected the diatom digestibility for post-larval abalone. Strongly attached diatoms such as *Cocconeis* spp. and *Achnanthes* spp. required a considerable strength to be detached from the substrata and were usually ruptured if dislodged. In contrast, many diatoms with a low adhesive strength were ingested without cell rupture, and the majority of the ingested cells passed through the gut alive and unbroken. There were some exceptions such as *Cylindrotheca closterium*, which had a low attachment strength, but showed a high digestion efficiency and induced rapid growth for post-larvae probably due to its structurally weak silica frustule.

Post-larvae may require diatom cell contents for rapid growth and abalone larger than 0.6-0.8 mm to begin to ingest *Cocconeis* spp. efficiently. These diatom species often dominate in the habitats of post-larval abalone both in the natural environment and on the pre-grazed plates used in abalone hatcheries. Benthic diatoms *Cocconeis* spp. are probably the main diet for larger post-larvae. In contrast, it has been suggested that juvenile abalone with a SL above 10 mm do not graze on *Cocconeis* species if more suitable foods are available (Ioriya and Suzuki, 1987; Suzuki *et al.*, 1987), because *Cocconeis* spp. are not efficient food sources for these large juveniles due to their small-volume cells and prostrate growth form (Takami *et al.*, 1996).

Changes from Diatom Feeding to Macroalgal Feeding

Large juveniles and adults of *H. discus hannai* prefer to feed on brown macroalgae of Laminariales (Kikuchi *et al.*, 1967; Sakai, 1962; Uki, 1981) and show rapid growth rates when fed these algal species (Kikuchi *et al.*, 1967; Uki, 1981; Uki *et al.*, 1986). Evidence from natural habitats suggests that the diet of the abalone is dominated by macroalgae as the juveniles grow (Shepherd and Cannon, 1988; Tomita and Tazawa, 1971). The dietary value of microscopic algal stages (juvenile sporophytes) of *Laminaria japonica* and the benthic diatom *Cylindrotheca closterium* for different developmental stages of *H. discus hannai* were compared to determine the size at which the abalone began to utilize macroalgae efficiently (Takami *et al.*, 2003).

Considerable variations were observed in the growth rates of the abalone between both algal types and the developmental stages of the abalone (**Fig. 3**), although most individuals were feeding actively. The post-larvae growth rates with a SL below 1.2 mm that were fed juvenile macroalgae of *L. japonica* were significantly lower than those of the post-larvae fed the benthic diatom *C. closterium*. In contrast, for post-larvae with a SL above 1.8 mm, feeding on juvenile macroalgae led to a significantly faster growth than those fed benthic diatom. Smaller post-larvae (< 1.2 mm SL) repeatedly grazed the surface of juvenile sporophytes without detaching these algae. Larger post-larvae (> 1.8 mm) could ingest large amounts of juvenile macroalgae. The dietary value of juvenile macroalgae for post-larval abalone depended on whether individuals could efficiently ingest algal fronds or not. The ingestion efficiency of post-larvae on algal diets was largely influenced by the radula morphology (see next section).



TAKAMI FIG. 3

Figure 3. Growth of post-larval *Haliotis discus hannai* (μm per day) fed juvenile sporophyte of *L. japonica* and benthic diatom *Cylindrotheca closterium* at four developmental stages. Each bar represents mean + SE. Data from Takami *et al.* (2003).

Although post-larvae fed the benthic diatom *Cylindrotheca. closterium* actively grazed and efficiently ingested diatom cells at all the post-larval stages, the relative dietary value of *C. closterium* decreased as post-larvae grew, compared with that of juvenile sporophytes of *L. japonica*. This implies that juvenile sporophytes provide a much higher biomass per unit area than small-volume, two-dimensional *C. closterium* films, if post-larvae are able to detach and ingest the juvenile sporophytes. *Cocconeis* films, which are suitable food sources for post-larvae > 0.8 mm in SL, also become energetically inadequate as abalone grow (Takami *et al.* 1996). The size at which the abalone begin to feed on macroalgae could be highly variable and probably depends on the macroalgal species, but it is apparent that *H. discus hannai* with a SL above 1.8 mm can utilize juvenile macroalgae *L. japonica* efficiently. Therefore the main food source may shift from a biofilm-dominated diet to a macroalgal-dominated diet from this size.

Possible Mechanisms Underlying the Feeding Changes

Ontogenetic Changes in Digestive Enzyme Activities

Brown macroalgae contain a significant amount of polysaccharides such as cellulose, alginate and laminarin. These polysaccharides are an important energy source for adult abalone which have high enzyme activities against these polysaccharides (Anzai *et al.*,

1991; Onishi *et al.*, 1985). Changes in the activity of the digestive enzymes for brown algal polysaccharides (carboxymethylcellulose, alginate, and laminarin) were measured in post-larval *H. discus hannai* at 7-46 days after the metamorphosis (Takami *et al.*, 1998). Enzyme activities were not detected in post-larvae about 0.5-0.6 mm SL, but by 1 mm SL, there was a detectable activity for all the enzymes (**Fig. 4**). In individuals > 1.5 mmSL, the total activities of the enzymes increased rapidly as they grew.

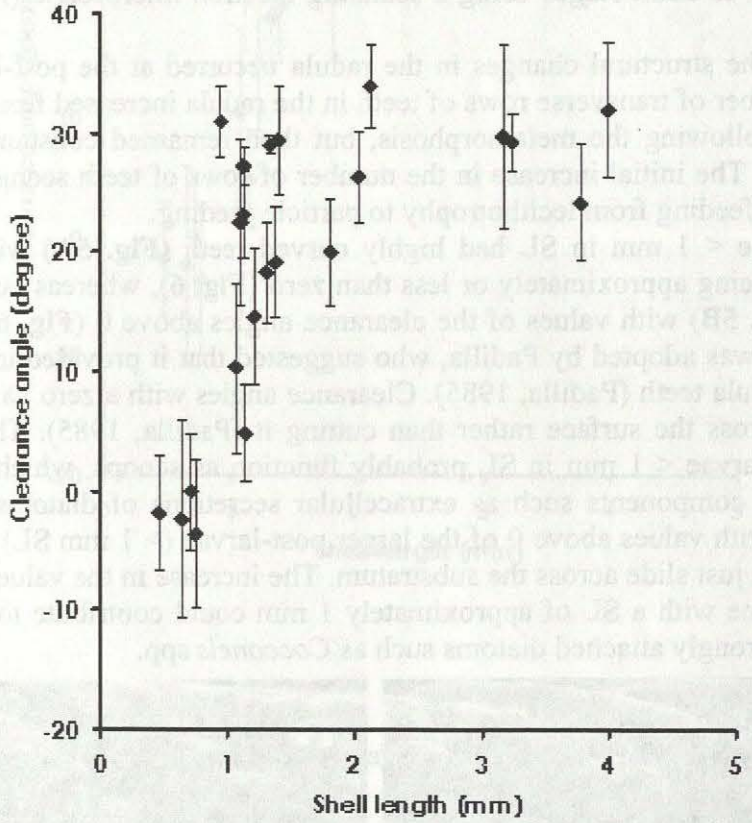


Figure 4. Relationship between total activities of digestive enzymes (μg RS/individual/h) and shell length (mm) in post-larval *Haliotis discus hannai.* Data from Takami *et al.* (1998).

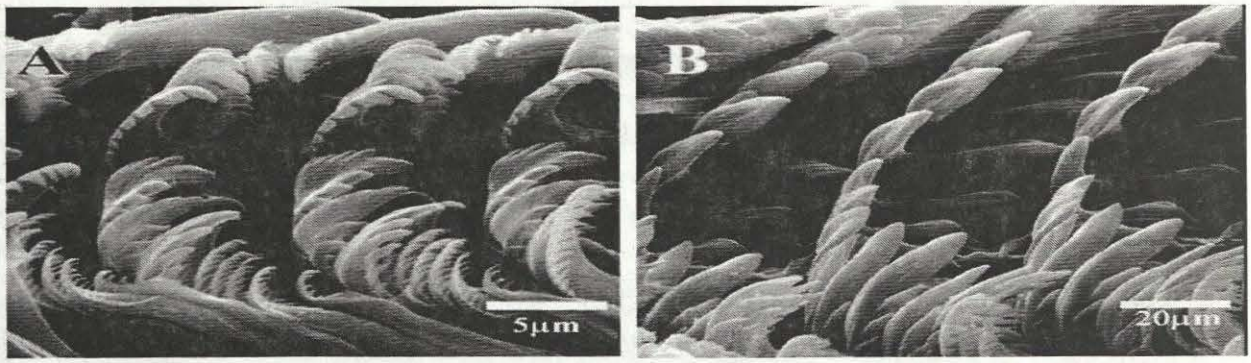
H. discus hannai with a SL above 1.8 mm began to utilize juvenile brown macroalgae as described above. The developmental process for the digestive enzyme activities shows that a series of enzymes useful for digesting brown algal polysaccharides is produced by post-larvae with a SL about 1 mm. This suggests that post-larvae with a SL about 1 mm may use brown algal polysaccharides if they can ingest either the algal cells or their surface biofilm.

Development of Radula Morphology

The post-larva's ability to ingest and digest the algae appears to be affected by the radula, as the abalone gut lacks any grinding mechanisms (Crofts, 1929). The developmental sequence of the radula morphology could be closely related to the changes in feeding (Roberts *et al.*, 1999b). Changes in the radula morphology were examined for *H. discus hannai* from larval to adult stages using a scanning electron microscope (Kawamura *et al.*, 2001).

Most of the structural changes in the radula occurred at the post-larval stage (< 4 mm SL). The number of transverse rows of teeth in the radula increased from 10-11 to 25-30 during the days following the metamorphosis, but then remained constant throughout the post-larval period. The initial increase in the number of rows of teeth seems to be related to the first change in feeding from lecithotrophy to particle feeding.

Post-larvae < 1 mm in SL had highly curved teeth (**Fig. 5A**) with values of the clearance angles being approximately or less than zero (**Fig. 6**), whereas larger abalone had straight teeth (**Fig. 5B**) with values of the clearance angles above 0 (**Fig. 6**). The clearance angle of the teeth was adopted by Padilla, who suggested that it provided information about the function of radula teeth (Padilla, 1985). Clearance angles with a zero value may result in a tooth sliding across the surface rather than cutting it (Padilla, 1985). The curved radula teeth of the post-larvae < 1 mm in SL probably function as scoops, which are suitable for collecting biofilm components such as extracellular secretions of diatoms and CCA. The clearance angles with values above 0 of the larger post-larvae (> 1 mm SL) may allow them to 'cut' rather than just slide across the substratum. The increase in the value of the clearance angle in the abalone with a SL of approximately 1 mm could contribute to the post-larva's ability to detach strongly attached diatoms such as *Cocconeis* spp.



TAKAMI FIG. 5

Figure 5. SEM photographs showing lateral views of the post-larval radula. A: Strongly curved rachidian and lateral teeth of post-larvae with a shell length of 0.72 mm on Day 11 post-settlement, with values of clearance angles near or less than zero. B: Radula teeth with clearance angles with values above 0 in a 3.24 mm post-larva on Day 63.
Data from Kawamura *et al.* (2000).

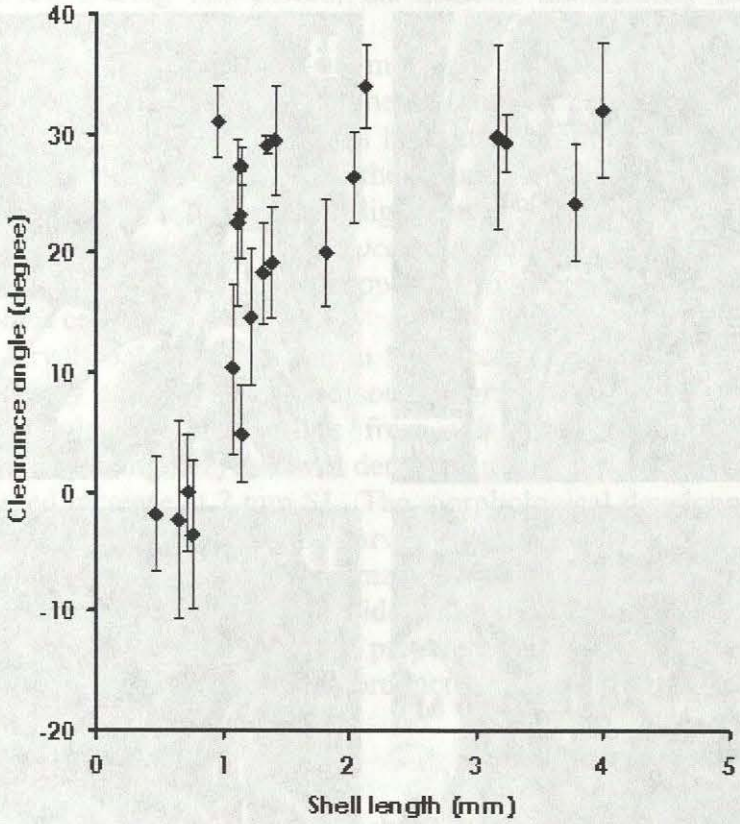


Figure 6. Relationship between value of clearance angle of teeth and post-larval shell length. Each data point shows the mean ± SE of 9-12 teeth on one radula. Data from Kawamura *et al.* (2000)

In post-larvae < 1 mm in SL, only 2 pairs of lateral teeth (L1, L2) were present in the radula (**Fig. 7A**). Three pairs of lateral teeth (L3-L5) were added gradually as the SL of the post-larvae increased from 0.9 mm to 1.9 mm (**Fig. 7B**). The serrations on the working edges of the rachidian (R) and lateral teeth became less pronounced as the abalone grew. Nearly all the serrations disappeared from the rachidian (R) and inner lateral teeth (L1, L2) when the SL was 2 mm, and the outer lateral teeth (L3-L5) became longer and more pointed (**Fig. 7C, D**). The reduction in tooth serrations suggests that the radula is less able to handle very small food particles such as bacteria and small diatoms, while the well developed L3-L5 teeth are more able to cut the macroalgae. These radula changes appear to be related to the changes in feeding habits from microbial to macroalgal diets.

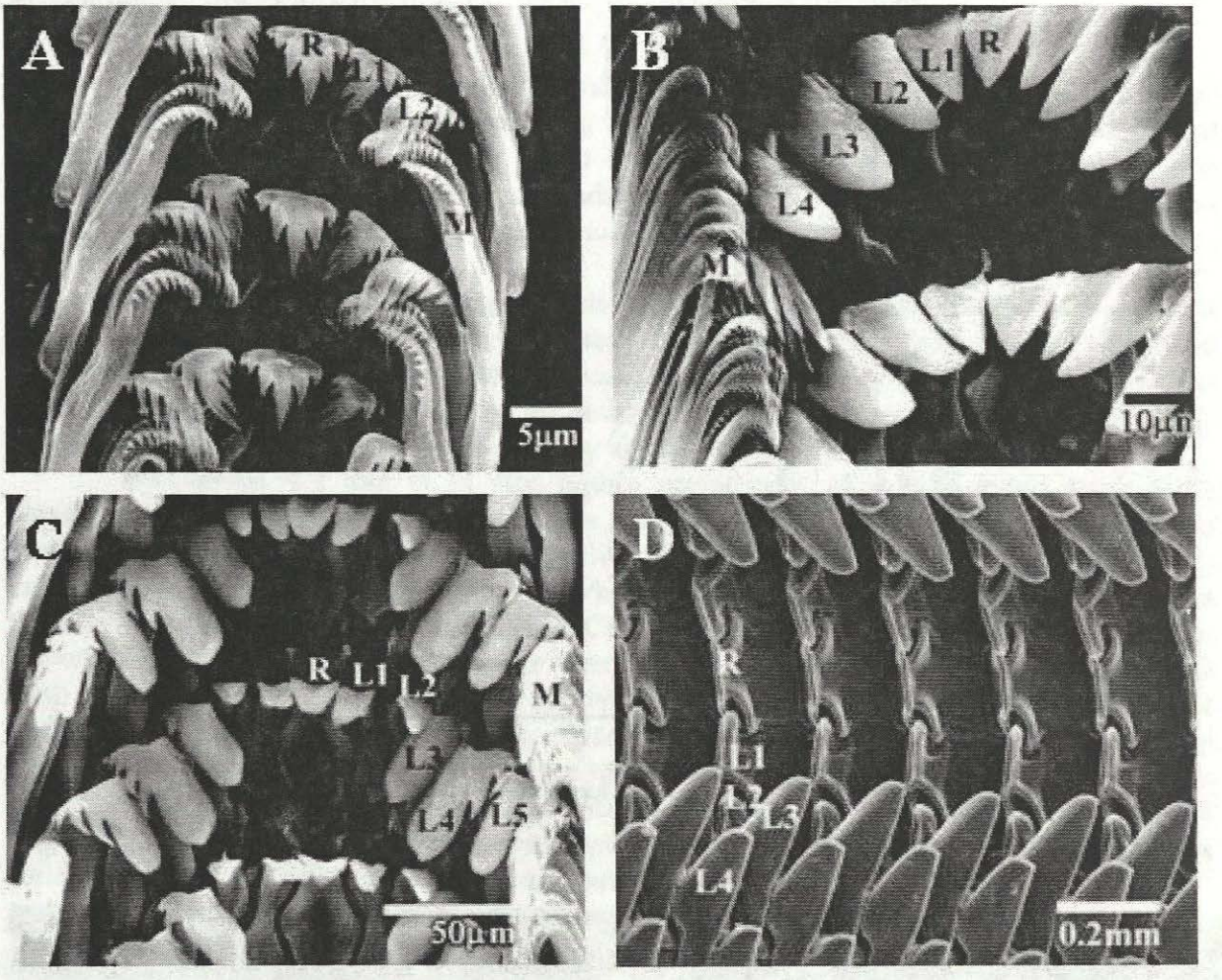


Figure 7. SEM photographs of the radula showing developmental stages. Radula formulae represent the numbers of teeth in a transverse row as follows: M+L+R+L+M (Voltzow 1994). R, Rachidian tooth; L1-L5, lateral teeth 1-5, M, marginal teeth. A: Post-larva 0.47 mm in shell length on Day 6 post-settlement, 3+2+R+2+3. B: Post-larva 1.9 mm in shell length on Day 49. Differentiation of the lateral teeth has started, ~15+(2+2)+R+(2+2)+ ~15. C: Post-larva 3.2 mm in shell length on Day 63. L3-L5 teeth are larger and longer than R, L1 and L2. ?+(3+2)+R+(2+3)+?. D: Juvenile 29.9 mm in shell length. L3-L5 teeth are much longer and more pointed than the central teeth (R, L1, L2). ?+(3+2)+R+(2+3)+?. Data from Kawamura *et al.* (2000).

Summary

Three major changes in the diet that were closely related to the developmental changes in the digestive enzyme activities, and radula morphology were identified as the abalone grew. The first change consisted of a shift from lecithotrophy (yolk absorption) to particle feeding. This change occurred around the time of metamorphosis with an overlap in

nutrition sources. During this period, the abalone immediately developed the radula to acquire an effective feeding organ.

Post-larvae with a SL of 0.6-0.8 mm were able to digest diatom diets and grew more rapidly on efficiently digested strains. The factors that control the digestibility of a diatom strain were complex, but diatoms with a high attachment strength such as *Cocconeis* spp., which often dominated on both CCA in the natural habitat and the pre-grazed plates used in the hatcheries, generally showed high digestion efficiencies. Post-larvae < 0.8 mm in SL were not able to efficiently detach *Cocconeis* cells from substrata. The morphological changes in the radula of the abalone appeared to contribute to the post-larva's ability to detach *Cocconeis* cells.

Post-larval abalone > 1.8 mm in SL became able to utilize juvenile macroalgae *L. japonica* efficiently and the main food sources gradually shifted from a biofilm-dominated diet to a macroalgal-dominated diet from this size. The activity of the macroalgal polysaccharide-degrading enzymes was detected in post-larvae with a SL of about 1 mm and showed a marked increase at 2 mm SL. The morphological development of the radula of *H. discus hannai* occurred mostly in post-larvae with a SL below 4 mm up to the level of the adult radula which was suitable for the grazing of macroalgae.

Food availability exerts a considerable impact on the survival and growth of post-larval *H. discus hannai*. It is important to prepare a suitable diet for each developmental stage of the abalone for constant and efficient production of hatchery seeds.

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Patterns, causes, and prevention of the mass mortality of juvenile blacklip pearl oyster Pinctada margaritifera (L.) cultured in Okinawa, subtropical Japan

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Key words
Blacklip pearl oyster, spat, mass mortality, subtropical Japan, infection

Abstract

The blacklip pearl oyster, *Pinctada margaritifera* (L.), provides many benefits for aquaculture in Okinawa and subtropical Japan. The spat have died off in massive numbers recently, possibly damaging the aquaculture industry. In the present study, the patterns, causes, and prevention of the mass mortality were investigated. Most investigations were made at 2 to 6 m depths in Kabira Bay, Okinawa, from September to December 2002.

Patterns: The spat were reared in perforated trays for 3 months starting in September 2002. Their cumulative deaths sharply increased starting at the end of October and finally reached and average death rate of 50.8 % while exceeding 90.5 % for half of the trays.

Causes: These deaths are attributable to species-specific factors of *P. margaritifera*, which may include pathogens rather than abiotic factors, predation, spat nutritional condition, or harmful phytoplankton, as indicated below. Only *P. margaritifera* spat showed rapid and massive death (mean cumulative mortality: 49.7 %), when reared together with the bivalves *Barbatia virescens* (Reeve) (17.2 %) and *Gafrarium tumidum* (Röding) (0.8 %), which suggests species-specific fatal factors. The abiotic factors (water temperature, salinity, turbidity, dissolved oxygen) showed no similar temporal fluctuation with the *P. margaritifera* cumulative mortality and/or were within safe ranges (20 to 28 °C, 32.5 to 34.7 PSU, 0.5 to 1.1 FTU, 7.0 to 9.1 mg / l). Predators killed only 2.2 % of spat on average, not accounting for the high cumulative mortality of spat. Malnutrition is unlikely to kill *P. margaritifera* spat quickly and massively, since most spat were found to endure starvation for > 70 days; and no serious malnutrition appears to occur, from sufficient chlorophyll *a* abundance (> 0.36 µg / l). The abundances of possibly-harmful phytoplankton showed no similar temporal fluctuations with the spat mortality and were within safe ranges (< 5 cells / ml). The existence of a pathogen is suggested from infection experiments: the *P. margaritifera* spat possibly infected with

pathogen in the sea were reared together with the spat that had been protected from any pathogen in the laboratory. This resulted in the mortalities of both spat groups. Although the sectioned tissue of the spat from the sea showed no identifiable pathogen during the mass-mortality season, it had deteriorated, and this suggests the symptoms of an infection.

Prevention: Pathogens and/or other fatal factors appeared to be influential between November 19 – 26, 2002. 21 groups of *P. margaritifera* spat were reared in the sea and then transferred on different days into the laboratory. Only those transferred after November 19, 2002 showed mortality. Another 21 groups were transferred from the labs to the sea. Only those transferred before November 26, 2002 showed mortality. The other spat from the sea were evacuated into the laboratory between November 19 –26, 2002, and these spat survived.

In conclusion, *P. margaritifera* spat begun to die massively from the end of October. This was probably a result of species-specific factors including pathogen, which can be prevented by evacuating spat into the laboratory for a short time during a dangerous period.

Introduction

The blacklip pearl oyster, *Pinctada margaritifera* (L.), produces black pearls, which provide many benefits (Shokita *et al.* 1991). The aquaculture techniques of *P. margaritifera* have been intensively studied since the beginning of 20th century. The spat of *P. margaritifera* can now be reared in the sea to produce black pearls. As a result, *P. margaritifera* is now cultured at many sites in the tropical and subtropical Indo - Pacific regions (Coeroli *et al.* 1984; Shokita *et al.* 1991).

There is, however, a problem. The spat of *P. margaritifera* are vulnerable to environmental stresses (Coeroli *et al.* 1984), and have died in massive numbers in the Solomon Islands, Tuticorin, India, and Marutea, French Polynesia. In the Solomon Islands, approximately 80 % of spat died during a 6-month period in some lantern nets due to predation by fish and invertebrates (Friedman and Southgate 1999). At Tuticorin, India, more than 80 % of spat died during a 45-day period in lantern nets for unknown causes (Alagarswami *et al.* 1989). At Marutea, French Polynesia, "heavy mortality" occurred due presumably to hypoxia (Sano 1998). The patterns and causes of the spat mortalities appear to differ between localities and thus should be investigated at each place.

In Okinawa, subtropical Japan, the spat of *P. margaritifera* appear to die off in massive numbers in some fish farms in recent years (Katsumata and Nakamori 2002, 2003). The spat were artificially produced in the laboratory, reared in trays in the fish farms, and showed mass mortality from September to November (local pearl producers, personal communication). These deaths appear to occur in only the fish farms, not laboratory tanks (Katsumata and Nakamori 2002). Hence, the mass mortality is attributable in part, at least, to some environmental factors specific to the fish farms. Yet, the patterns, causes, and prevention of the mass mortality have not been fully investigated thus far.

In the present study, we first clarify the mortality patterns of *P. margaritifera* spat unique to a fish farm in Okinawa (Chapter 1). We then reveal the causes of the mortality (Chapter 2). Finally, we develop prevention methods.(Chapter 3).

1. Patterns

Aims

In this chapter, we reveal the patterns of the day-to-day and tray-to-tray variations in cumulative mortality of *P. margaritifera* spat.

Material and Methods

The study site is Kabira Bay (24° 27.3' N, 124° 8.7' E) at Ishigaki Island, Okinawa, subtropical Japan **(Fig. 1).** To this bay, pearl producers usually transfer *P. margaritifera* 1 month after an artificial hatch in July through September. They rear *P. margaritifera* at 2 to 6 m depths in lantern nets, trays, and panel nets that are suspended from headlines near the sea surface.

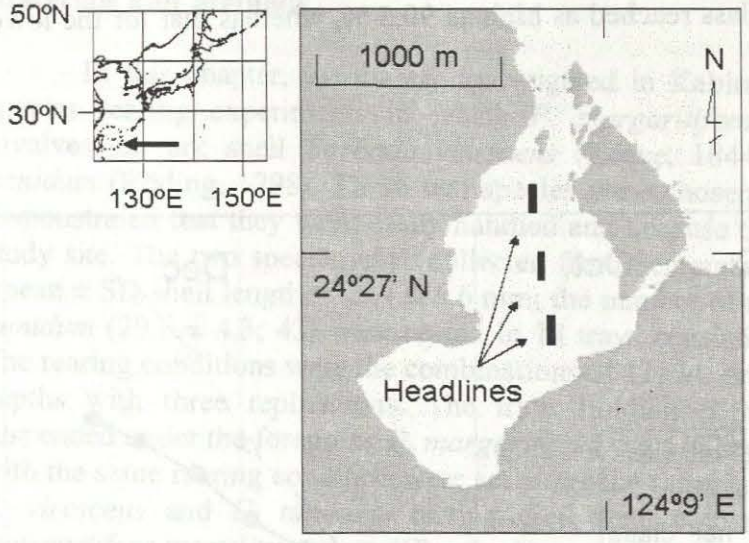

Figure 1. Maps of Kabira Bay.

In the observation of mortality pattern, *P. margaritifera* spat were artificially produced from wild parents. The spat were reared in the laboratory tanks, fed living *Chaetocerous* sp. (> 5,000 cells / ml). On 13th September 2002, 3456 spat with mean ± SD dorsoventral measurement (Sims 1993) being 14.5 ± 2.4 mm were transferred to the study site. These spat were kept in perforated trays with a lid (33 cm W × 21 cm L × 8 cm H; covered with a 2 mm × 2 mm mesh). Rearing conditions were varied among the trays. There were either 12, 24, 36, 72 or 144 spat placed in each tray. Trays were suspended at depth of either 2 or 6 m. The number of cells dividing spat was either one or four. Such variations between trays resulted in no significant difference in among the cumulative mortality rate (Kurihara *et al.* submitted). For each rearing condition, 3 trays were prepared (60 trays in total) and suspended from different headlines **(Fig. 1)**. Under each headline, the trays were arranged in a random order at 1.4 m intervals in a horizontal direction. On a boat, these trays were

checked weekly for the count of dead spat and were washed for the removal of fouling until 19th December 2002. The dead spat and predators (ranellid gastropods, portunid crabs, polyclads) were removed.

The cumulative mortality for each survey date was calculated for the spat in each tray according the following formulas: 100 × (cumulative number of individuals having died until the date) / (total number of individuals at the start of survey). The cumulative mortality was averaged across: 1) all 60 trays; 2) the 30 trays in the upper-half class in which cumulative mortality was the 1st to 30th on the last survey date; 3) and the 30 trays in the remaining, lower-half class. Such calculations for different classes can show a tray-to-tray variation in cumulative mortality.

Results

The averaged cumulative morality averaged across all trays sharply increased on October 31st, finally reaching 50.8 % (**Fig. 2**). Such a sharp rise was caused by rapid increases in cumulative mortality by only a few trays. On the last survey date, the mean mortality for the upper-half class reached as high as 90.5 %, whereas that for the lower-half class remained only 11.1 %.

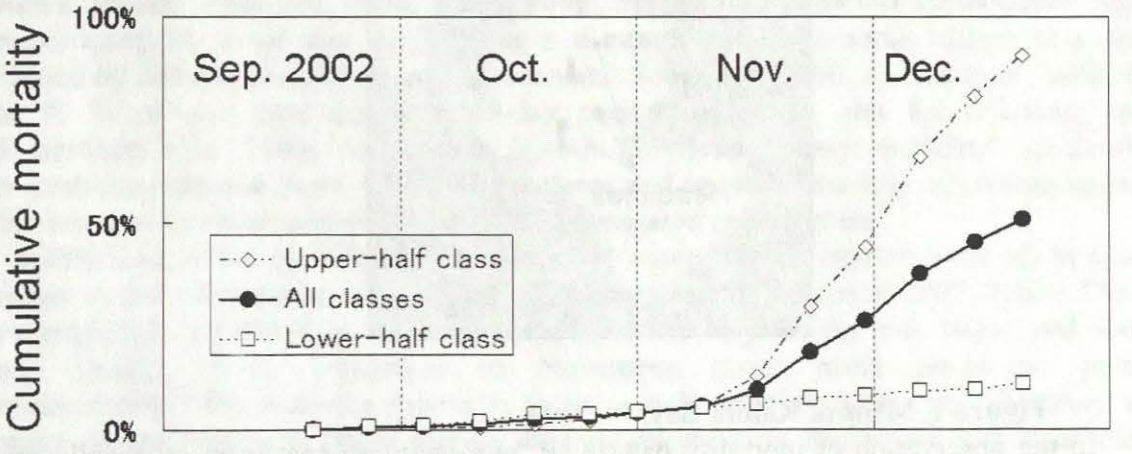

Figure 2. *Pinctada margaritifera*. Mean cumulative mortalities (%) for trays in the lower half, upper-half, and all classes.

Discussion

Both the average and tray-to-tray variation of *P. margaritifera* spat mortality are much higher in the present study site than in previous study sites (Friedman and Southgate 1999; Southgate and Beer 2000). Further, the mortality rate in some trays increased nearly 90 % within 2 months, and such rapidness had never been reported previously (Alagarswami *et*

al. 1989; Friedman and Southgate 1999; Southgate and Beer 1997, 2000). The next chapter explores why such massive and rapid mortality occurred in only a few trays

2. Causes

Aims

In this chapter, we examine the causes of the mass mortality found in *P. margaritifera* spat. First, we examine whether only *P. margaritifera* spat died off in massive numbers among several bivalve species. If so, the *P. margaritifera* mortality may be due to species-specific factors, and not to more general factors such as harmful phytoplankton (Parry *et al.* 1989; Fukuyo *et al.* 2002). We then examine the factors that have been related with the mass mortalities of cultured bivalves (Ford 1992; Burreson and Calvo 1996; Friedman and Southgate 1999; Fukuyo *et al.* 2002; Gosling 2003), namely: (a) abiotic factors (water temperature, salinity, turbidity and dissolved oxygen); (b) predation; (c) nutritional condition of spat; (d) harmful phytoplankton; and (e) pathogen.

Materials and Methods

In this chapter, we mainly investigated in Kabira Bay. First, we conducted three-species rearing experiments in which *P. margaritifera* were reared with filter-feeding bivalves, an ark shell *Barbatia virescens* (Reeve, 1844), and a Venus clam *Gafrafium tumidum* (Röding, 1798). These two species were chosen because preliminary experiments demonstrated that they were easily handled and because the species were abundant near the study site. The two species were collected from September 5-9, 2002. Each of *B. virescens* (mean ± SD shell length = 23.4 ± 6.6 mm; the number of measured individuals = 63) and *G. tumidum* (29.6 ± 4.3; 43) were reared in 18 trays consisting of one cell with no sediments. The rearing conditions were the combinations of 12, 24, or 36 individuals per tray at 2 or 6 m depths with three replications. The trays holding *B. virescens* and *G. tumidum* were suspended under the foregoing *P. margaritifera* trays (Chapter 1) in a way that the three trays with the same rearing condition were set along the same vertical rope (termed as "3-Sp set"). *B. virescens* and *G. tumidum* having died were counted during the observation of *P. margaritifera* mortality pattern (Chapter 1).

The water temperature, salinity, and turbidity were measured at 2 and 6 m depths near the center of each headline (**Fig. 1**) simultaneously with the observation of *P. margaritifera* mortality pattern (Chapter 1). They were determined by censors "Compact-CT" and "Compact-CL" (Allec Co. LTD. http://www.alec-electronics.co.jp/) with the accuracies of ± 0.02 °C, ± 0.02 PSU, and ± 2 FTU. Dissolved oxygen was measured at 3 m depth near the center of the central headline at 30-minute intervals from 10th October to 24th December 2002. These measurements used the "Compact-DOW" (Allec Co. LTD.) with the accuracy of ± 0.001 mg / l.

The mortality of *P. margaritifera* spat due to predation was evaluated for each tray. Dead *P. margaritifera* whose shells were attached or probably broken by predators (i.e., ranellid gastropods and portunid crabs) were counted and compounded with observations of *P. margaritifera* mortality pattern (Chapter 1). Cumulative predation rate for each date was

calculated as: 100 × (cumulative number of individuals having died of predation until the date) / (total number of individuals at the start of survey).

To examine whether malnutrition of *P. margaritifera* spat caused the mass mortality, various investigations were conducted. First, the abundance of phytoplankton, the food of *P. margaritifera* spat, was estimated from chlorophyll *a* abundance (μg / l), measured at 2 and 6 m depths near the center of each headline (**Fig. 1**). This measurement concurrently compared the observation of the *P. margaritifera* mortality pattern (Chapter 1), using a Compact-CL with the accuracy of ±0.02 μg / l. Further, the nutritional condition of *P. margaritifera* was estimated from glycogen content, RNA/DNA ratio, and the relative weight of digestive organ. The higher these indices are the better the nutritional condition may be. Each index was measured at 3 to 7 day intervals from September 17 to December 3, 2002. On each day, the glycogen content (i.e., the ratio of glycogen weight to soft-tissue wet weight; ‰) and RNA/DNA ratio (the ratio of RNA weight to DNA weight) were measured following the methods described in Okumura *et al.* (2002) and references therein for 20 oysters. Of these, 10 oysters had been kept at 3 m depth in Kabira Bay, and the remaining 10 had been reared in tanks in the laboratory since September 12, 2002. The relative weight of the digestive organs (= wet weights of stomach and style sac / wet weight of all soft tissue) was measured for 10 one year old *P. margaritifera* that had been kept at 3 m depth in Kabira Bay since 12th September 2002. In addition to these investigations, starvation experiments were conducted to examine how long *P. margaritifera* spat can endure starvation. 10 *P. margaritifera* spat were kept from July 22 - November 19, 2002 in each of four aquariums (35.0 cm W × 21.5 cm L × 26.0 cm H) in the laboratory. The spat were fed living *Chaetocerous* sp. (> 5,000 cells / ml) in two aquariums, whereas others starved in the remaining two. Seawater wsa added to these aquariums and regulated at 25 °C or left natural. The mortalities of the spat were checked every day.

The abundance of possibly harmful phytoplankton (Parry *et al.* 1989; Fukuyo *et al.* 2002) was estimated by sampling 5 l sampled of seawater at 3 m depth near the central headline (**Fig. 1**) at the intervals of 3 to 7 days between September 17 - December 24, 2002. The seawater sampled was preserved with 5% formalin and filtered through 10-μm mesh. The sample remaining on the mesh was identified to the lowest possible taxonomic level. The phytoplankton was found to consist of as many as 42 taxonomic groups (mainly species). Of these, only possibly - harmful phytoplankton were analyzed, after grouped at either family level (Prorocentraceae, Gymnodiniaceae, Peridiniaceae, and Rhizosoleniaceae) or order level (when family-level identification was difficult; Gymnodiniales and Peridiniales).

Infection experiments were conducted to examine whether some infectious causing fatality factors could spread from spat to spat. They were carried out in 2001 (Run 1) and 2002 (Run 2) Each year 10 to 20 possibly infected spat were taken from the sea just after *P. margaritifera* spat died off in massive numbers in Kabira Bay. They were transferred into either 4 or 7 aquariums in the laboratory with running seawater. We also transferred 10 to 60 spat that had been kept in the laboratory (thus, unlikely to be infected by pathogen) into each aquarium. Only when some of the spat from the sea were infected by pathogen would both groups from the sea and laboratory die. Thus, mortalities of both groups would be positively correlated. The spat were kept in aquariums for 30 to 40 days. Their mortality rates during such different periods were unified by calculating mortality per 35 days according to Akçakaya et al. (1999). In addition to the infection experiments, the soft tissue of spat was

observed for symptoms of infection by pathogen. Spat were sampled from a fish farm in Urasoko Bay (24°27' N, 124°13' E) near Kabira Bay just before and after the massive kills on October 8 and 12, 1999. The spat were preserved in Davidson's Solution. The sections of its gills were stained with hematoxylin-eosin for the preparation of histological slides.

Results

In the three-species rearing experiments, the cumulative mortality of *P. margaritifera* showed species-specific fluctuation patterns (**Fig. 3**). Mortality rates sharply increased after October 31st, finally getting very high: the means within the lower-half class, across all trays, and within the upper-half class were 10.7 %, 49.7 %, and 88.7 %, respectively. In contrast, the cumulative mortality of *B. virescens* slowly increased after October 11 and finally remained low (4.1 %, 17.2 %, and 30.4 %). The cumulative mortality of *G. tumidum* remained very low throughout the experiments (0.0%, 0.8%, and 1.6%).

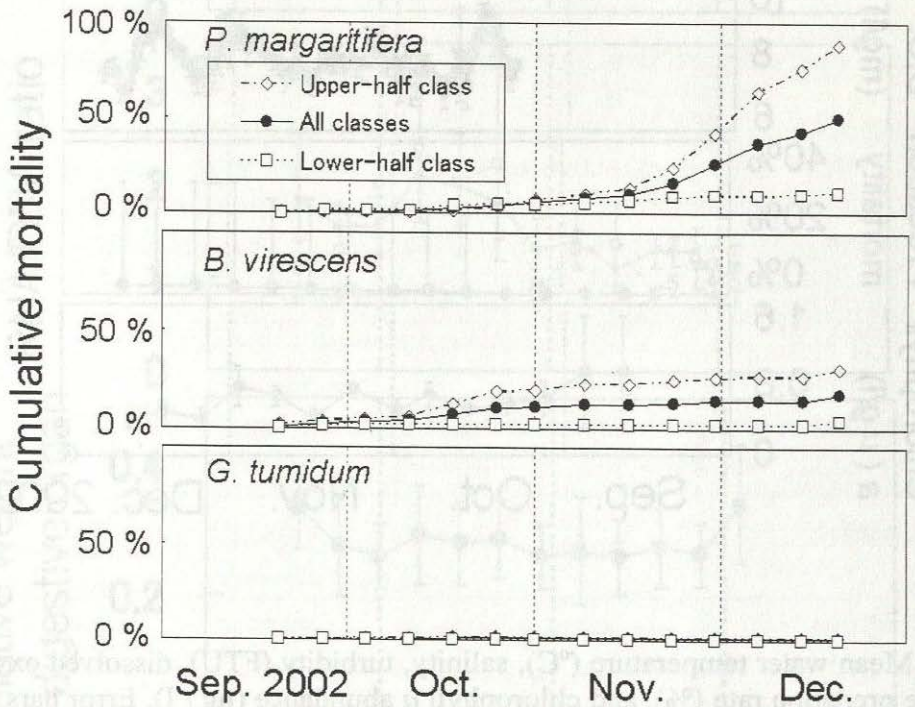

Figure 3. *Pinctada margaritifera, Barbatia virescens, and Gafrarium tumidum*. Mean cumulative mortalities (%) for trays in the lower-half, upper-half, and all classes.

Among abiotic environmental factors (**Fig. 4**), only the water temperature correlated with the sharp increase in *P. margaritifera* cumulative mortality rates after the end of October (**Fig. 2**). From this period, the mean water temperature decreased from 26.3 °C to 20.2 °C (max.: 28.1 °C on 4th October). After the same period, the averages of salinity (33.6

to 34.4 in range during the study period), turbidity (0.5 to 1.1 FTU), dissolved oxygen concentrations (7.0 to 9.1 mg / l) repeatedly increased and decreased.

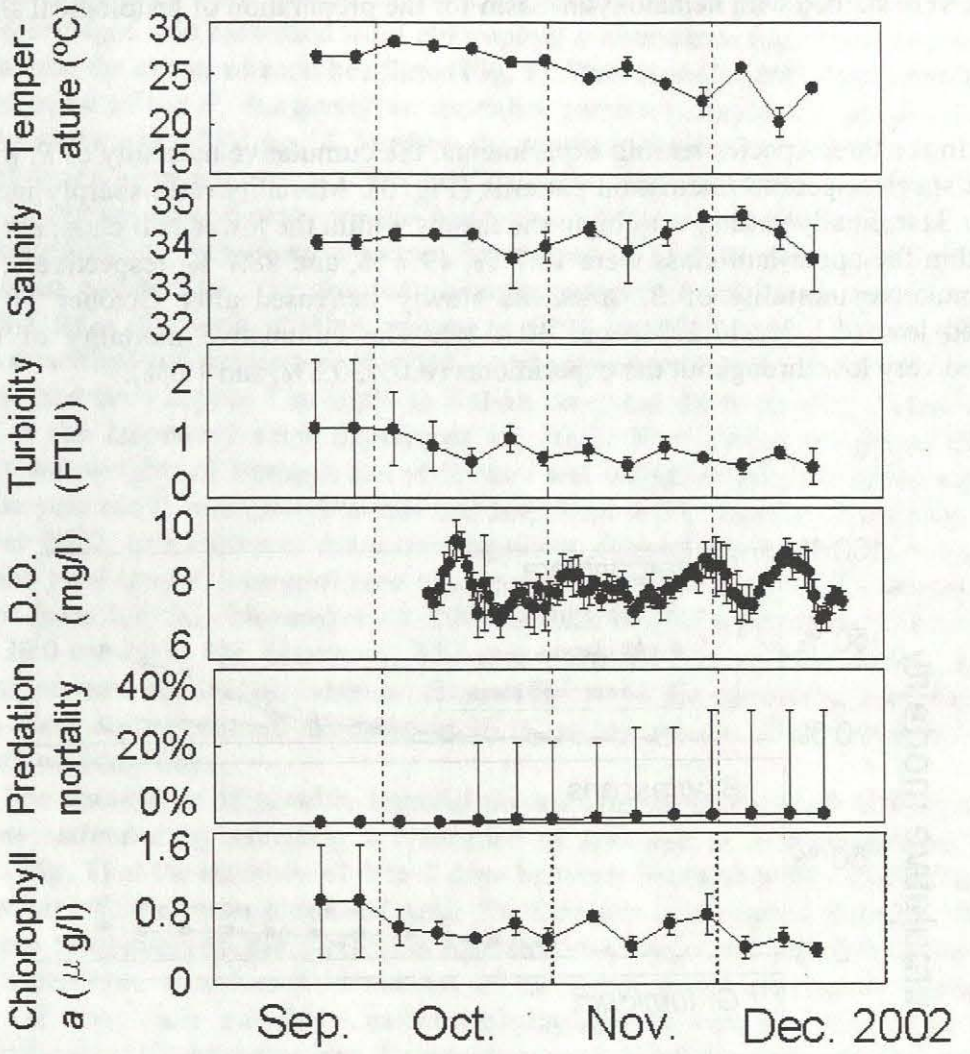

Figure 4. Mean water temperature (°C), salinity, turbidity (FTU), dissolved oxygen (mg / l), cumulative predation rate (%), and chlorophyll *a* abundance (μg / l). Error bars denote either [maximum minus mean] of predation mortality rate or ± 1 SD of the other factors.

Cumulative predation rate (**Fig. 4**) did not correspond to the sharp increase in *P. margaritifera* mortality. The average of cumulative predation rate slowly increased from 11th October, namely, before the outbreak of *P. margaritifera* mass mortality. On the last day of the survey, it remained only 2.2 %, and the maximum was only 29.2 %.

The mean abundance of chlorophyll *a* repeatedly increased and decreased (range: 0.36 to 0.88 μg / l; **Fig. 4**), not corresponding to the sharp increase in *P. margaritifera* cumulative mortality after the end of October. On the other hand, the indices of *P. margaritifera*

nutritional condition (**Fig. 5**) corresponded somewhat to the *P. margaritifera* mortality pattern. That is, glycogen content and RNA/DNA ratio of the *P. margaritifera* in Kabira Bay gradually decreased from 8th or 15th October to 3rd December. And, relative weight of digestive organ remained low between 24th September and 26th November. In the starvation experiments (**Fig. 6**), it took at least 70 days under each temperature condition for the mortality of starved *P. margaritifera* to exceed the mortality of fed *P. margaritifera* by > 20 %.

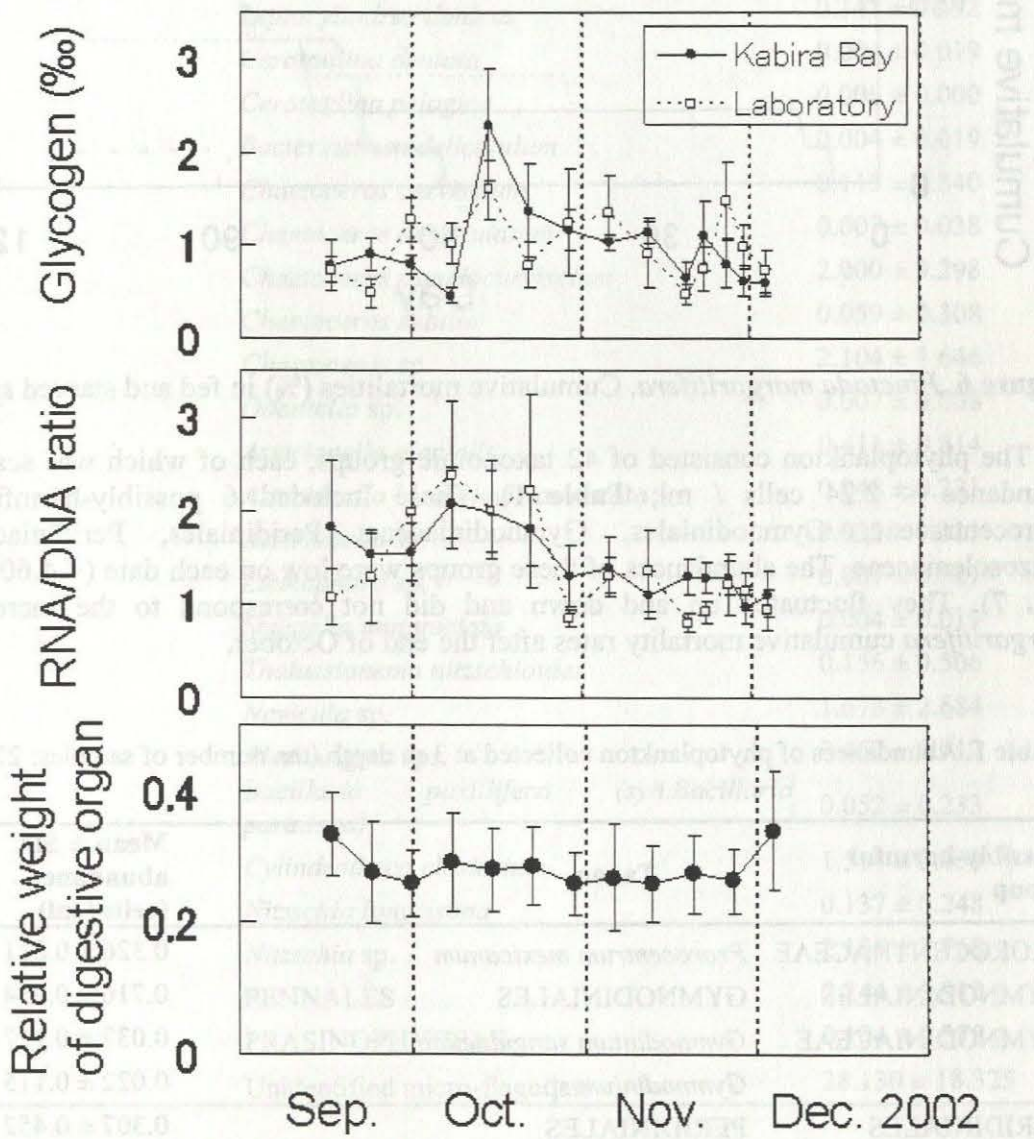

Figure 5. *Pinctada margaritifera*. Mean ± SD glycogen content (‰), RNA/DNA ratio, and relative weight of digestive organ.

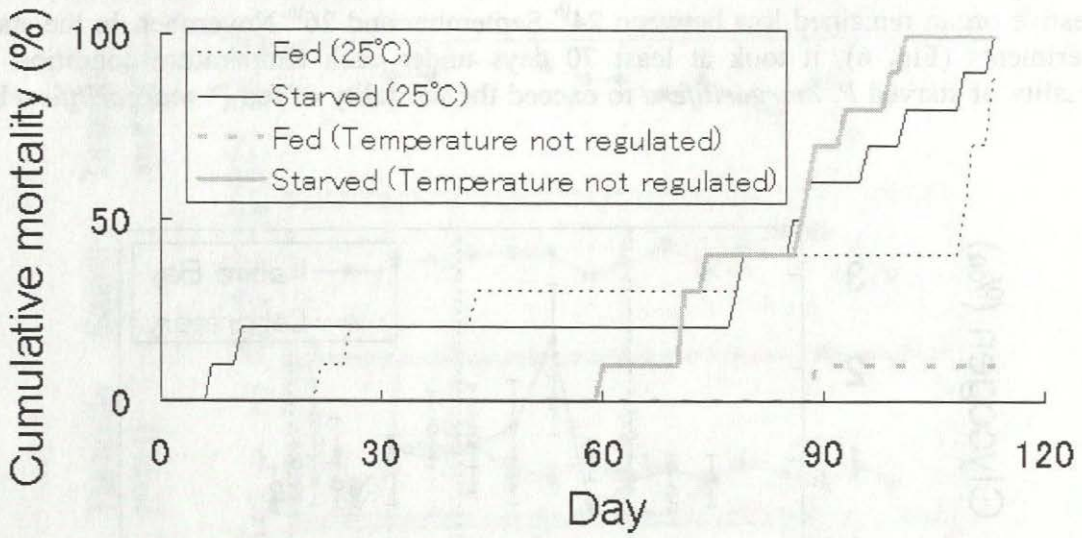

Figure 6. *Pinctada margaritifera*. Cumulative mortalities (%) in fed and starved spat.

The phytoplankton consisted of 42 taxonomic groups, each of which was scarce (mean abundance < 2.24 cells / ml; **Table 1**). These included 6 possibly-harmful groups: Prorocentraceae, Gymnodiniales, Gymnodiniaceae, Peridiniales, Peridiniaceae, and Rhizosoleniaceae. The abundances of these groups were low on each date (< 4.60 cells / ml; **Fig. 7**). They fluctuated up and down and did not correspond to the increase in *P. margaritifera* cumulative mortality rates after the end of October.

Table 1. Abundances of phytoplankton collected at 3 m depth (the number of samples: 27)

Possibly-harmful group	Taxon	Mean ± SD abundance (cells / ml)
PROROCENTRACEAE	*Prorocentrum mexicanum*	0.326 ± 0.811
GYMNODINIALES	GYMNODINIALES	0.716 ± 0.954
GYMNODINIACEAE	*Gymnodinium sanguineum*	0.037 ± 0.157
	Gymnodinium sp.	0.022 ± 0.115
PERIDINIALES	PERIDINIALES	0.307 ± 0.452
PERIDINIACEAE	*Protoperidinium bipes*	0.004 ± 0.019
	Protoperidinium oceanicum	0.004 ± 0.019
	Protoperidinium sp.	0.199 ± 0.395
RHIZOSOLENIACEAE	*Rhizosolenia alata (Proboscia alata)*	0.015 ± 0.046
	Rhizosolenia imbricata	0.230 ± 0.637

	Rhizosolenia stolterfothii (Guinardia striata)	0.122 ± 0.345
	Rhizosolenia sp.	0.015 ± 0.053
Others	Oscillatoriaceae	0.196 ± 0.313
	CRYPTOMONADALES	1.727 ± 2.843
	Distephanus speculum var.octonarius	0.004 ± 0.019
	Thalassiosira sp.	0.322 ± 0.879
	Thalassiosiraceae	0.393 ± 0.680
	Leptocylindrus danicus	0.141 ± 0.692
	Cerataulina dentata	0.004 ± 0.019
	Cerataulina pelagica	0.000 ± 0.000
	Bacteriastrum delicatulum	0.004 ± 0.019
	Chaetoceros curvisetum	0.115 ± 0.340
	Chaetoceros denticulatum	0.007 ± 0.038
	Chaetoceros pseudocurvisetum	2.000 ± 9.298
	Chaetoceros subtile	0.059 ± 0.308
	Chaetoceros sp.	2.104 ± 1.646
	Odontella sp.	0.007 ± 0.038
	Asterionella glacialis	0.411 ± 0.814
	Asterionella bleakeleyi var.notata	0.048 ± 0.231
	Asterionella sp.	0.022 ± 0.115
	Licmophora sp.	0.007 ± 0.027
	Striatella unipunctata	0.004 ± 0.019
	Thalassionema nitzschioides	0.156 ± 0.506
	Navicula sp.	1.678 ± 2.684
	Pleurosigma sp.	0.467 ± 0.912
	Bacillaria paxillifera (syn.Bacillaria paradoxa)	0.052 ± 0.233
	Cylindrotheca closterium	1.514 ± 3.031
	Nitzschia longissima	0.137 ± 0.248
	Nitzschia sp.	2.156 ± 3.768
	PENNALES	2.244 ± 1.913
	PRASINOPHYCEAE	0.104 ± 0.378
	Unidentified micro-flagellate	28.130 ± 18.325

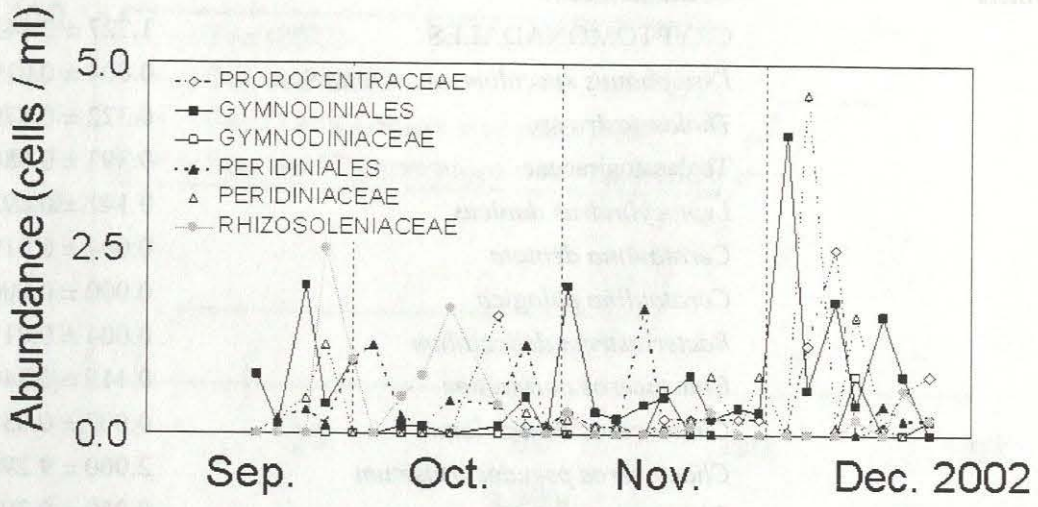

Figure 7. Abundances (cells / ml) of possibly - harmful groups of phytoplankton

In both runs of the infection experiment, the *P. margaritifera* collected from the sea and those having been kept in the laboratory showed positive correlations with the mortality rates (**Fig. 8**). A *P. margaritifera* had a normal tissue just before the breakout of mass-mortality, whereas after the breakout it possessed a deteriorated tissue consisting of shrunken cells without any recognizable pathogen (**Fig. 9**).

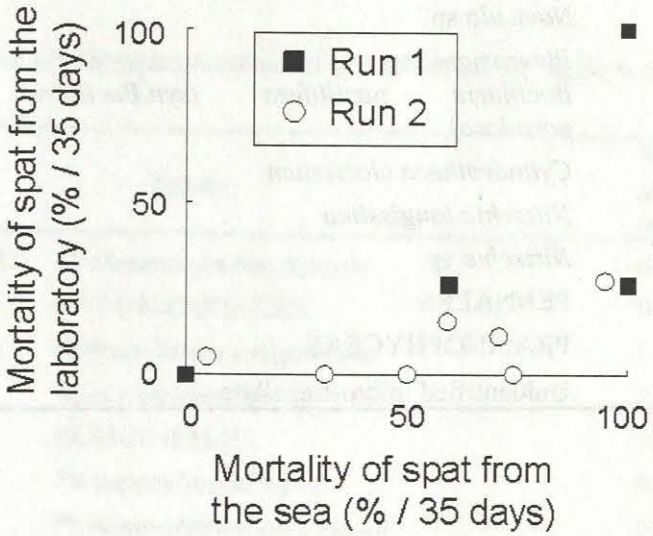

Figure 8. *Pinctada margaritifera*. Mortalities (% / 35 days) of spat groups from the sea and laboratory, presented for Runs 1 and 2 in the infection experiments.

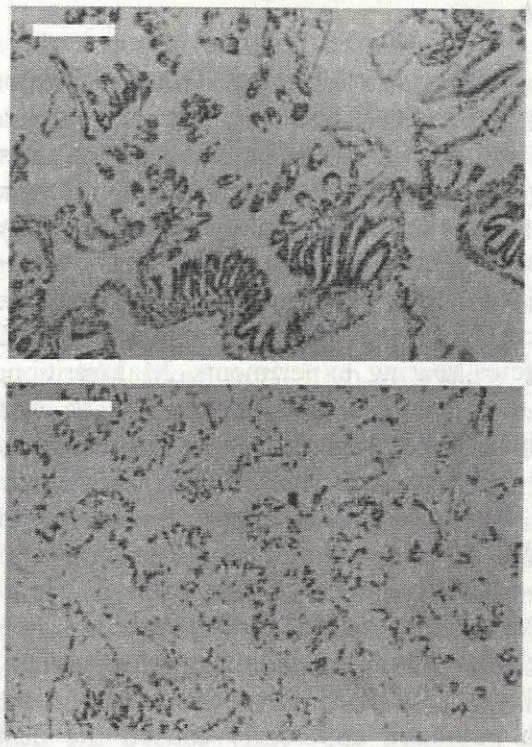

Figure 9. *Pinctada margaritifera*. Gills in spat before and after the outbreak of mass mortality (upper and lower panels, respectively). Scale bar: 1 mm.

Discussion

The mass mortality of *P. margaritifera* spat appears to be due mainly to its species-specific factors. This is suggested by the three-species rearing experiments in which the cumulative mortality of only *P. margaritifera* sharply increased after the end of October, finally getting very high in nearly half the trays. Although such species-specific factors might be intensified by some non-specific stresses (as suggested from the death of some *B. virescens* reared with *P. margaritifera*), the non-specific stresses themselves are somewhat unlikely to explain the sudden and massive death of only *P. margaritifera*.

Such species-specific fatal factors for *P. margaritifera* are unlikely to include abiotic environmental factors. Although the water temperature showed a similar pattern of temporal fluctuation with the cumulative mortality of *P. margaritifera*, its direct influence on the mass mortality is unlikely. This conclusion is drawn from the range of water temperature during the study period, 20 to 28 °C, in which *P. margaritifera* seldom died in laboratory tanks (see Chapter 3). In addition, the range was approximately within the optimum ranges regarding bioenergetic terms (23 to 28 °C; Yukihira *et al.* 2000) and oxygen consumption (15 to 33 °C; Sugiyama and Tomori 1988). The salinity showed a temporal fluctuation dissimilar to the mortality fluctuation. Further, it was within the range of 32.5 to 34.7, in which *P. margaritifera* is reported to survive in the present study site (25.0 to 34.5: Katsumata and Nakamori 2002). The turbidity fluctuation was dissimilar from the mortality fluctuation. The

dissolved oxygen was also dissimilar and, in addition, appears to be sufficient for shellfishes to survive (> 7.0 mg /l).

None of predation, possibly-harmful phytoplankton, and malnutrition may appear to have directly killed many *P. margaritifera* spat either. Predators killed only 2.2 % of *P. margaritifera* on average, not accounting for the high cumulative mortality of *P. margaritifera* (mean: 50.8 %). The abundances of possibly-harmful phytoplankton temporally changed in dissimilar manners from the *P. margaritifera* mortality. In addition, they were far lower (< 5 cells / ml) than the abundance of a phytoplankton species previously reported to kill bivalves (> 50 to 20,000 cells / ml: Parry *et al.* 1989; Matsuyama *et al.* 1997, 1998; Nagai *et al.* 2000). Further, although harmful phytoplankton kills many bivalve species simultaneously in general (Parry *et al.* 1989; Fukuyo *et al.* 2002), such phenomena were not found in the three-species rearing experiments. Malnutrition may not directly lead to the mass mortality of *P. margaritifera* spat either. This is because food abundance may be sufficient for the survival of *P. margaritifera*, as suggested by the chlorophyll *a* abundance exceeding 0.36 μg / l (Vacelet *et al.* 1996). Further, malnutrition may not quickly kill *P. margaritifera* spat, as suggested by the long-term survival of experimentally starved spat. Yet, it should be noted that the nutritional condition indices of the spat were low during the *P. margaritifera* mass-mortality period. The nutritional condition might perhaps be so low that the spat would be vulnerable to some fatal factors.

In comparison with the foregoing factors, pathogens are more likely to kill many *P. margaritifera* spat. This is indicated from the infection experiments in which the spat possibly infected with pathogen apparently caused the death of the other spat through infection. Pathogens can only properly explain the rapid and massive death of spat found a part of trays (Chapter 1). Pathogen may easily spread within only a part of trays that held infected spat because to the closed environment and the aggregation of spat with byssus (Southgate and Beer 2000). Although no pathogens were identified, the existence of pathogens could also explain the deteriorated tissue found in spat during the mass-mortality period. However, the results of the infection experiments should be carefully interpreted. A priori environmental factors might be much worse in one aquarium than in another aquarium. Thus in the former aquarium each spat might die more easily, and this would lead to apparent infection from spat to spat. Even if a dead spat has no pathogen, the dead body itself might do damage to the other spat in the same aquarium through, say, perishing the water, which may also lead to apparent infection.

3. Prevention

Aims

The sharp increase in cumulative mortality from the end of October (Chapter 1) might indicate that pathogens and/or other fatal factors are influential within only a short period. If so, one might be able to prevent the mass mortality by evacuating *P. margaritifera* spat from the sea to a safer place during this short period. In this chapter, we first examine whether pathogen and/or other fatal factors are influential only for a short period. We then examined whether evacuation during such a period is effective to avert the mass mortality. We evacuated the spat into the laboratory tanks with filtered seawater because local pearl producers through personal communications have suggested this to be safe for the spat.

Material and Methods

We conducted the transference experiments to determine when *P. margaritifera* spat begin to be exposed to danger in the sea (the first experiment) and when the vulnerability ends (the second experiment). In the first experiment, 21 groups of spat (46 to 50 individuals / group) had been reared at 3 m depth at the central headline (**Fig. 1**) in Kabira Bay starting on September 12, 2002. Each group was evacuated into the laboratory aquariums on different days at nearly weekly intervals from September 17, 2002. These evacuated groups continued to be reared in the aquariums until December 24, 2002 or later, and their cumulative mortalities were determined. If it was too late to evacuate a group from risk factors occurring in Kabira Bay, the group would show very high mortality. Hence, by examining the evacuation dates from which spat groups begun to show high mortality, the beginning of dangerous period may be estimated. In the second experiment, the spat were transferred in the opposite direction of the first experiment. Twenty-one groups of spat (12 to 50 individuals / group) had been reared in the laboratory since its birth. Each group was transferred to 3 m depth in Kabira Bay on different days at nearly weekly intervals from September 17, 2002. These transferred groups were reared in Kabira Bay until February 13, 2003, and their cumulative mortalities were determined. If a group were transferred too early to Kabira Bay while fatal factors were still influential, such a group would show very high mortality. Hence, by examining the transference dates results the end of dangerous period may be estimated.

We conducted evacuation experiments, considering the results of the above-mentioned experiments that the dangerous period may be November 19-26, 2002 (see Results). In this period only, four trays holding 12 to 38 spat were evacuated in aquariums in the laboratory. Except for this period, the trays were suspended at 3 m depth in Kabira Bay from September 12, 2002 - February 13, 2003. The cumulative mortality was estimated for each tray. For each experiment, cumulative mortality was unified by calculating mortality per 100 days according to Akçakaya et al. (1999).

Results

Of the *P. margaritifera* groups evacuated from the sea to the laboratory, only those evacuated after November 19th showed high mortality frequently (22.4 to 95.6 % / 100 days; **Fig. 10**). Of the groups transferred in the opposite direction, only those transferred before 26th November died often (6.6 to 77.6 % / 100 days). Thus, November 19-26 was the period within which the *P. margaritifera* groups transferred in either direction died was. All of the additional four spat groups evacuated around this period survived.

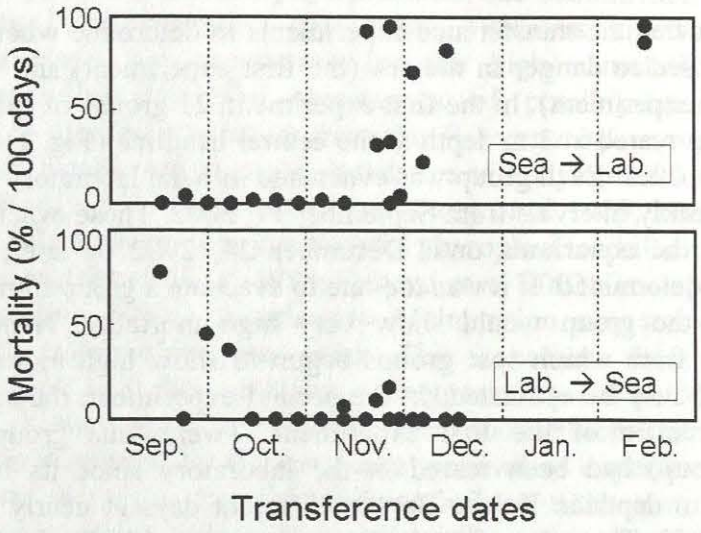

Figure 10. *Pinctada margaritifera*. Mortalities (% / 100 days) of spat groups transferred from the sea to the laboratory (upper panel) and from the laboratory to the sea (lower panel).

Discussion

The transference experiments and evacuation experiments suggest that fatal factors existed between November 19-26, approximately. During this period, fatal factors such as pathogens may be greatly influential in the sea, and/or their effects may be intensified by the other factors such as malnutrition of *P. margaritifera* spat (see Chapter 2). Such a dangerous period is likely to differ between years and between sites in Kabira Bay. That is, during yrs 2000 to 2002 the spat began to die off in massive numbers at the end of October (Chapter 1; Katsumata and Nakamori 2002, 2003), whereas before then they had started to die earlier, such as the beginning of October (Kurihara, personal data; local pearl producers, personal communication). In trays within a 10 to 100 m distance from each other, the spat started to die with a difference of 30 days at maximum (Katsumata and Nakamori 2003; Kurihara, personal data). Hence, considering such annual and spatial variations in dangerous periods, one should evacuate spat for a long period that is inclusive of November 19-26. To determine the evacuation period, one should monitor spat mortality in a part of the trays every autumn at various sites in Kabira Bay. If some of the trays begin to show high mortality, one should evacuate the other trays into the laboratory; and if monitored trays stopped showing mortality, one may as well return the evacuated trays to the sea.

Acknowledgments

We acknowledge the advice of Drs. K. Ashida, I. Imai, S. Oka, Y. Shimadzu, S. Tabata, K. Tamai, and the staff of Okinawa Prefecture Fisheries Experimental Station and Seikai National Fisheries Research Institute. We thank Drs. M. Katoh, K. Kiso, H. Yamada,

and the local pearl producers for their help in the study site. We are financially supported by the SNFRI Project Fund.

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Relationship Between Environmental Food and Glycogen Contents in Pen Shells

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Key words: pen shell, glycogen content, environmental food, and sexual maturation

Abstract

We investigated several factors influencing changes in the glycogen content in pen shells *Atrina pectinata* and made a standard health index for glycogen content of pen shells living in Ariake Bay. Seasonal variation of glycogen content and sexual maturation was determined as followed: (February, 2000 ~ February 2001) the glycogen content increased in adductor muscle from February-April (70～80mg/g), but decreased gradually following the development of reproductive glands, showing 15mg/g in spawning season (July ~ September). After spawning season (October ~ December), low-levels (3mg/g) of glycogen content were observed and rose to 13mg/g following high availability of phytoplankton (January). The fluctuating glycogen content could be related to reproductive gland development, as glycogen is an energy source needed for life and reproductive gland development. Results were achieved the following measures for the influence of feed quantity on environment. For experiment 1, "Tolerance to food deprivation", specimens were fed *Chaetoceros* sp. for 4 days after sampling and divided into fed group and unfed group. Changes of glycogen content in the unfed group decreased continuously (12mg/g to 2mg/g over one week) with specimens dying after day 7 until all had died by day 15. However, the glycogen content of the fed group remained at 10～12mg/g with a survival rate of 80%. For experiment 2, "recuperation from unfed conditions", pen cells were used in this study 2 months after spawning. This experiment was divided into two groups: one group was starved for one week and the other for one month. Recuperation of glycogen intake for 40 days was observed thereafter. Both groups differed in tolerance for food deprivation. The one week group accumulated glycogen rapidly (2 to 20mg/g over one week), but the one-month group did not (2 to 12mg/g over 1 month). The one month group survival rate was lower (70 %) than in the one-week group (100%). These results suggest that the pen shell survival rate is influenced by short-term lack of food in spawning season. Also, pen shells after spawning season can accumulate glycogen rapidly in the presence of high food availability after spawning season. Therefore, it is suggested that pen shells had low glycogen content (3mg/g) in Ariake Bay during autumn 2000 due to low phytoplankton levels over several weeks.

Introduction

The pen shell *Atrina pectinata* grows up to a shell length of 20-25cm (**Fig. 1**) and lives in a sandy mud bottom under tidal areas in bays at a depth of 5-20m. The pen shell is an expensive food in western Japan. However, pen shell fishing production has been decreased significantly in recent years in Ariake Bay, Kyushu Island (**Fig. 2**). Pen shell fishing production has been less than 1,000 tons in Ariake Bay since 1999 and comprised about 90% of domestic fishing production the until early 1980 (Okutani 1997). It was reported that pen shell stocks have decreased due to mass mortality in the growth stage in recent years (Matsui 2002, Kawahara and Ito 2003).

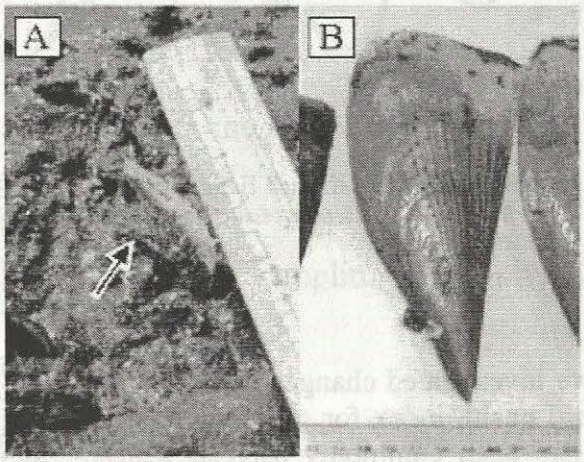

Fig.1 Environmental conditions and pen shell morphology
A: Pen shell living in tidal land B: Pen shell caught in Ariake Bay

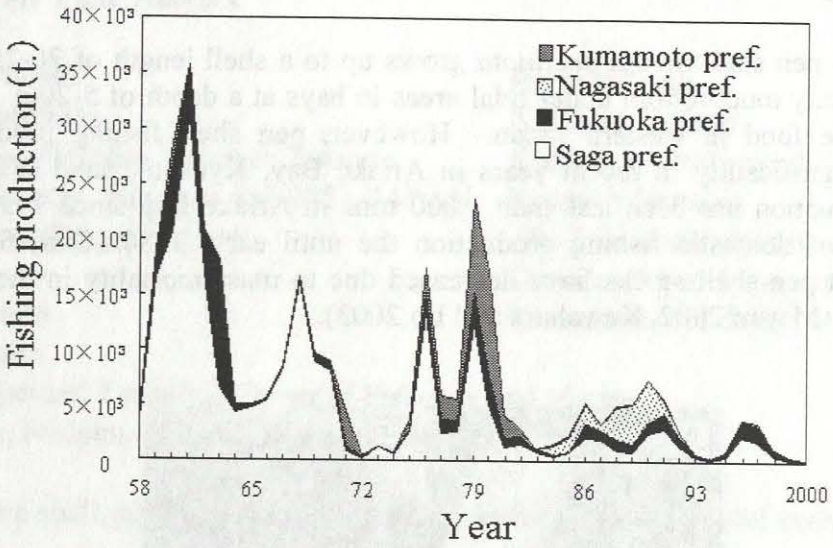

Fig.2 Annual changes of pen shell fishing production in Ariake Bay
(Data from annual prefectural fisheries production statistical reports)

In this study, we investigated changes in factors affecting glycogen content in pen shells and made a standard health index for glycogen content of pen shells living in Ariake Bay. Glycogen content has been commonly used as a health index for bivalves such as scallops *Patinopecten yessoensis* (Miyazono and Nakano 2000, Yamanaka 2002), oysters *Crassostrea gigas* (Akashige and Fushimi 1992, Mori *et.al.* 1965, Yamamura and Watanabe 1964), pearl oysters *Pinctada martensii* (Shinomiya *et.al.* 1997, Uchimura 1999) and Manila clams *Ruditapes philippinarum* (Takagi and Shimizu 1963).

Materials and Methods

Seasonal Variation of Glycogen Contents and Sexual Maturation

Two types of pen shell *Atrina pectinata* were found in Japan in a recent study. Yokogawa (1996) distinguished them according to shell type (non-scaly vs. scaly form) and isozymic patterns. In this study, we used scaly form pen shells, because pen shells of this type are the main objects of fishing in Ariake Bay.

Pen shells that were of the same age group cohort occurring in 1999 were collected every month (February, 2000-February, 2001) by helmet-type diving in the fishing grounds of Ariake Bay (**Fig. 3**). Adductor muscle was analyzed for glycogen content by the Anthron method (Kamada and Hamada 1985) and the organization block was isolated from reproductive glands, fixed in 10% formalin solution, embedded in paraffin, cut into thin sections, stained with HE (Hematoxylin and Eosin) and observed with an optical microscope.

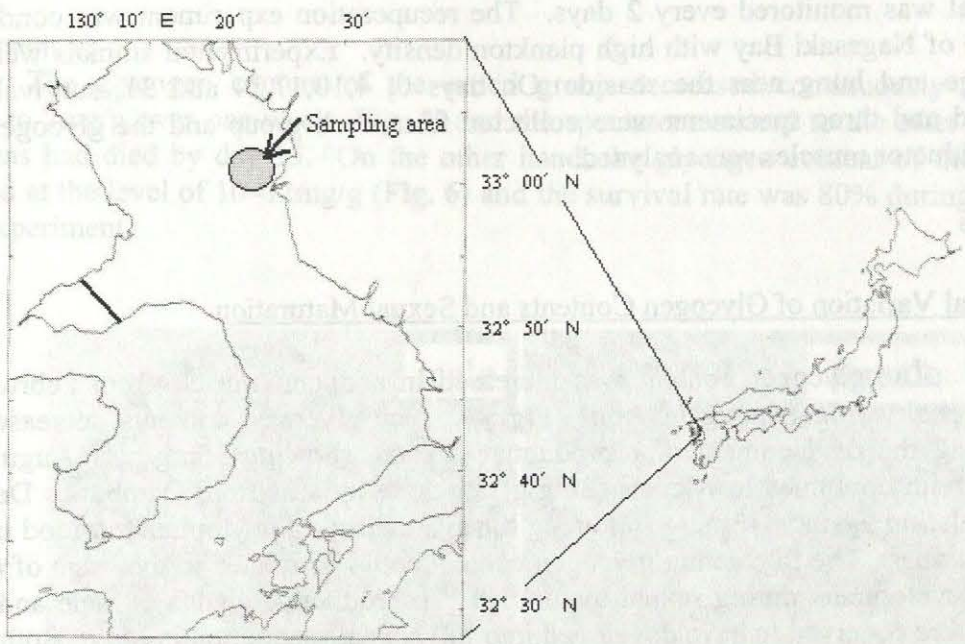

Fig.3 Location of sampling area in Ariake Bay

The classification of sexual maturation stages in shellfish has already been reported in Scallops *Patinopecten yessoensis* (Mori *et al.* 1977), Umitake *Barnea dilatata* (Yamasaki 1993) and Manila clams *Ruditapes philippinarum* (Toba *et.al.* 1993), thus we compared our results of pen shells with these previous studies.

Influence of Changes in Glycogen Contents According to Feed Quantity Environment

Experiment 1: Tolerance for Food Deprivation

Pen shells collected in tidal lands of Ariake Bay during spawning season were used for the experiment. Specimens fed phytoplankton (*Chaetoceros* sp.) for 4 days after sampling were divided into two groups, fed and starved. The experiment was conducted in 50L containers containing sandy mud collected in Ariake Bay, water temperature 24.5 ~ 26.5℃, salinity 31.0 ~ 32.5PSU, and specimens were fed *Chaetoceros* sp. 18×10^6cells/day (for fed group). Both groups were observed for changes in the survival rates and glycogen content over two weeks. During the experiment, three specimens of each group were analyzed for adductor muscle glycogen content on days 0, 2, 5, 9, and 13.

Experiment 2: Recuperation From Unfed Conditions

Specimens were collected in Ariake Bay from September to October. Before taking the samples, we took some specimens to check their sexual stage. During this time, pen shells were already spent or the spawning season was already finished. The animals were brought to the laboratory and kept in a 100L aquarium. The animals were divided into

two groups. One group was starved for 1 week and another for 1 month respectively. Survival was monitored every 2 days. The recuperation experiment was conducted in the seaside of Nagasaki Bay with high plankton density. Experimental animals were put inside net cage and hung near the seaside. On days 0, 4,10,17,24 and 31, survival rates were checked and three specimens were collected from each group and the glycogen content in their adductor muscles was analyzed.

Results

Seasonal Variation of Glycogen Contents and Sexual Maturation

The glycogen content was increased in adductor muscle from February to April with a peak of 70-80mg/g in April (**Fig. 4**). The glycogen contents decreased gradually following the development of reproductive glands, showing 15mg/g in August spawning season with continued low-levels (3mg/g) for three months from October to December and accumulating again to 13mg/g following the availability of phytoplankton food in January of the next year. The fluctuating glycogen content could be related to the stage of reproductive gland development during sexual maturation. Reproductive glands of male and female pen shells were observed to have developed into full sexual maturation in May. Both phenomena were well correlated.

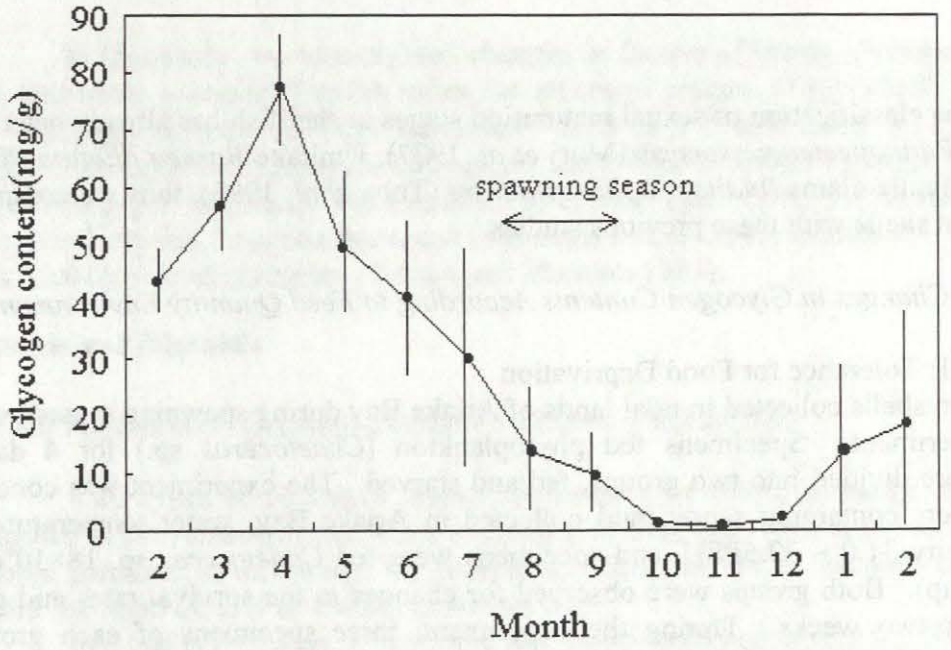

Fig.4 Seasonal changes of glycogen content in pen shells.

Influence of Changes in Glycogen Content by Feed Quantity Environment

(Experiment 1)

The glycogen content of the unfed group decreased continuously from about 12mg/g to 2mg/g over one week (**Fig. 5**) and the specimens started to die after day 7. All specimens had died by day 15. On the other hand, the glycogen content of the fed group remained at the level of 10~12mg/g (**Fig. 6**) and the survival rate was 80% during the course of the experiment.

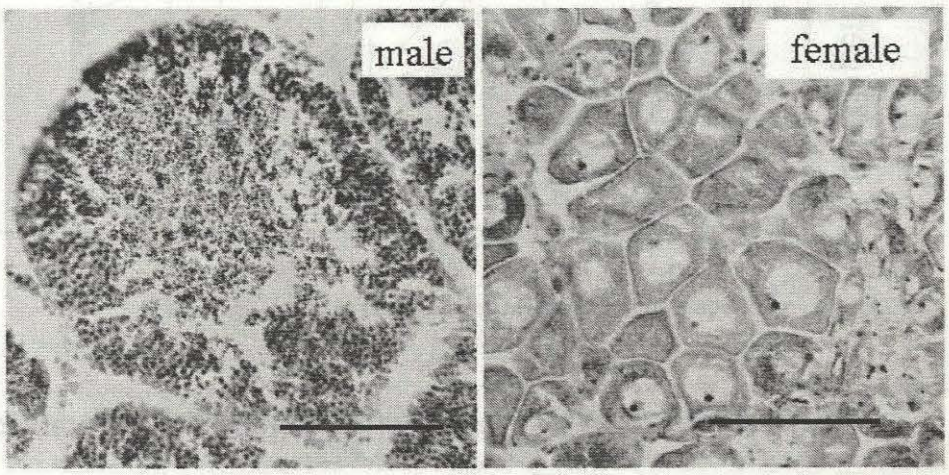

Fig.5 Histological observation of gonads of pen shells in May stained with HE (Hematoxylin and Eosin) . bars 50 μ m.

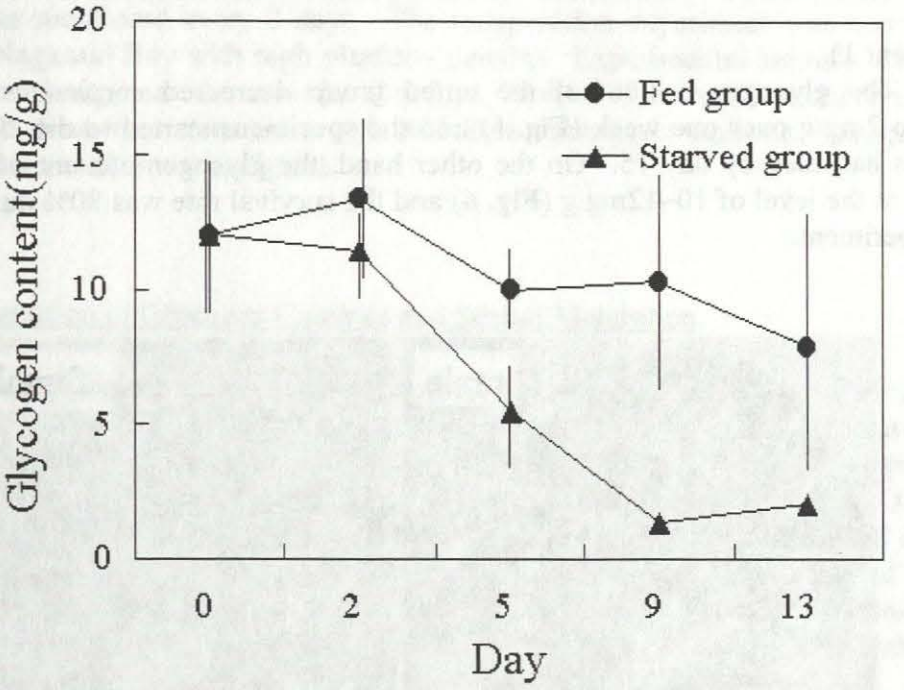

Fig.6 Comparison of glycogen content between fed and starved group

(Experiment 2)

No mortality was observed in the group starved for one week, while in the group starved for one month, mortality was 15% during the period before the experiment commenced. During the experiment, the survival rate of the one-month group (70%) was lower than the one-week group (100%). Both groups showed differing recuperation processes. The one-week group accumulated glycogen rapidly from 2mg/g to 20mg/g over a one week period while the one-month group couldn't accumulate glycogen rapidly, accumulating glycogen from 2mg/g to 12mg/g over a one month period (**Fig. 7**).

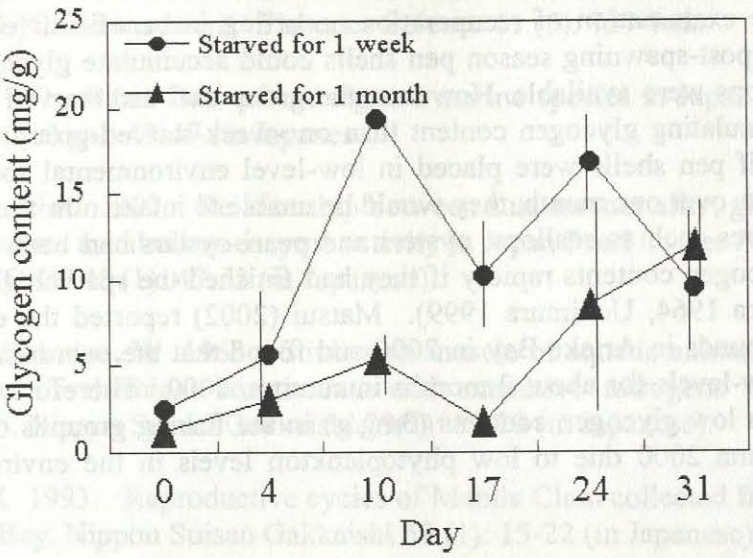

Fig.7 Glycogen content of pen shells during recuperation

Discussion

Seasonal changes of glycogen content in pen shells are deeply influenced by the development of reproductive glands. Glycogen contents were decreased gradually leading up to the spawning season. It was thought that this phenomenon is similar to the seasonality of glycogen content of scallops (Yamanaka 2000), oysters (Yamamura and Watanabe 1964) and pearl oysters (Uchimura 1999). In this study, the glycogen contents in pen shells started to decrease when the reproductive glands entered sexual maturation in May. It is suggested that pen shells rapidly consume glycogen during sexual maturation stage because glycogen is well known as an energy source needed for both life and reproductive gland development.

Numaguchi (1995) investigated tolerance for lack of food for pearl oysters. He reported the relationship between survival rate and glycogen contents in pearl oysters, observing that the survival rate became low when the glycogen contents were about 3mg/g. In this study, we investigated the tolerance for food deprivation in pen shells and found that survival rate lowered when the glycogen contents in adductor muscle indicated about 2mg/g. Matsui (2002) surveyed the relationship between survival rate and environmental conditions in the fishing grounds of pen shells in Ariake Bay, 2000. He found the survival rate became gradually decreased during the summer to autumn, 2000. We analyzed the glycogen contents in adductor muscle of pen shells that were caught in the same place during the same period and found the glycogen contents were about 3mg/g during autumn. These results suggest that the survival rate decreased when the glycogen contents approximated only 2~3mg/g in adductor muscle of pen shells. On the other hand, it is considered that pen shell survival rate

and glycogen content in spawning season is influenced by a 2-week period of food deprivation.

In the examination of recuperation occurring in beneficial feeding conditions, it was found that post-spawning season pen shells could accumulate glycogen rapidly if good feeding conditions were available. However, the group that had starved for one month was slower in accumulating glycogen content than one-week-starved-group. This phenomenon suggested that if pen shells were placed in low-level environmental food conditions for a long time such as over one month, they would be unable to intake nutrition rapidly.

Bivalves such as scallops, oysters and pearl oysters had been already reported to accumulate glycogen contents rapidly if they had finished the spawning season (Yamanaka 2002, Yamamura 1964, Uchimura 1999). Matsui (2002) reported the environment of pen shell fishing grounds in Ariake Bay in 2000 and found that the occurrence of plankton was continued at low-levels for about 2 months in autumn, 2000. Therefore, it is suggested that pen shells had a low glycogen contents (3mg/g) in the fishing grounds of Ariake Bay for 3 months in autumn 2000 due to low phytoplankton levels in the environment for several weeks.

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IV. Sturgeon Culture and Other

Non-Invasive Surgery Techniques in Fish Research: A Review on Esophageal Intubation, Dorsal Aorta Cannulation, and Urinary Catheterization in Sturgeon

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Abstract

The combined technique consisting of esophageal intubation, dorsal aortal cannulation and urinary catheterization has been shown to be an effective technique for the oral administration of a compound, repeated blood sampling, and continuous urinary collection while minimizing the stress response associated with handling and sampling. The successful use of the combined technique warrants further research for refinement and expansion of the technique to include additional sampling routes to better account for clearance pathways not currently accounted for. The combined technique can be adapted for use in studies of nutritional, toxicological, physiological, or therapeutic agents of importance.

Introduction

Esophageal intubation, dorsal aorta cannulation, and urinary catheterization techniques have been used singly or in combination to study the biology of many species of fish. Different aspects of surgical techniques such as anesthetics, cannulation, confinement, post-operational recovery, etc. have been reviewed by Summerfelt and Smith (1990), Houston (1990), Iwama and Ackerman (1994), Axelsson and Fritsche (1994), and Horsberg (1994). The objective of the present paper is not to review extensively or exhaustively any single technique, but to focus on the combination of the above techniques and possible inclusion of other techniques in future research using sturgeon as a model species.

Fitting of an indwelling tube into the mouth (Holeton and Randall 1967, Wood and Randall 1973), esophagus (Carrick and Balment 1983, Glover and Hogstrand 2002), or intestine (Youson et al. 1988) has been used to deliver various compounds into the gastrointestinal tract (GIT) of different species of fish. The intubation method allows quantitative delivery of specific compounds at multiple doses and at different time intervals with minimum regurgitation, disturbance, and stress to the fish.

Dorsal aorta cannulation was developed by Conte et al. (1963) and Smith and Bell (1964), and modified and improved by Houston (1971) and Soivio et al. (1972, 1975). The technique has been modified, adapted, and used to monitor blood/plasma parameters in Atlantic salmon, common carp (Kayama and Iijima 1976), tuna (Jones et al. 1986), channel catfish (Mazik, 1994), striped bass (Cech et al. 1996), tilapia (Ron et al. 1995), charr (Haug and Hals 2000), and grouper (Lo et al. 2003) with minimum stress. Other vascular cannulation techniques including ventral aorta (Kirsch 1972), caudal arterial and venous (Watters and Smith 1973), ventricle (Thorpe and Ince 1974), afferent and efferent vessels of gill arches (Wells et al. 1984), sinus venous (Ishimatsu et al. 1988), hepatic portal vein (McLean and Ash 1988), and pneumogastric artery and vein (Hyde and Perry 1989)

cannulations have been developed for different species of fish. These techniques have also been used singularly or in combinations in different species of fish.

Urinary bladder catheterization has been used to study the kidney and urinary bladder functions in fishes (Wood and Patrick 1994) including lungfish (Sawyer 1966), rainbow trout (Holmes and Stainer 1966), American eel (Butler 1969), southern flounder (Hickman 1968), and common carp (Kakuta et al. 1986). The esophageal intubation, vascular cannulations, and urinary catheterization have been used individually and in combination to study the dynamics of specific compounds in fishes under different treatment and environmental conditions. Data obtained with the combined techniques are especially suited for estimating kinetic parameters in models describing a particular biological process or metabolic dynamics of a specific compound (Schultz and Hayton 1991. and Kleinow 1991).

Validation and Uses of Combined Technique in White Sturgeon

Force-feeding different carbohydrates in gelatin capsules were used as an oral challenge test in white sturgeon (Hung 1991). Various vascular cannulations were used to study the blood/plasma responses of Adriatic (Randall et al. 1992, Di Marco et al. 1999), white (McEnroe and Cech 1985, Crocker and Cech 2000, Crocker et al. 1998), and green (Belanger et al. 2001) sturgeon to different treatments and environmental conditions. Urinary catheterization of white sturgeon was used to monitor urinary free amino acid after an oral dose of a free amino acid mixture (Ng et al. 1996).

The first simultaneous use of dorsal aorta cannulation, urinary catheterization, and esophageal intubation in the white sturgeon was described by Deng et al. (2000). This study was carried out in order to assess the effectiveness of the combined technique to quantitatively deliver nutrients, sample blood repeatedly, and continuously collect urine while minimizing the handling stress associated with force-feeding and blood sampling. The quantitative delivery of nutrients through the esophageal intubation tube was confirmed by the complete recovery (105±5%) of an intubated dose of Cr_2O_3, an inert unabsorbable marker, in the GIT. Repeated blood sampling and collection of urine was also achieved with the absence of a stress response during sampling of the experimental fish.

Stress levels, determined by the levels of cortisol and glucose in the plasma up to 72 h post-operation, returned to basal levels within 48 h post operation and remained at basal levels through 72 h post-operation (Deng et al. 2000). The basal cortisol levels determined by Deng et al. (2000) were in agreement with those reported in Rainbow trout (Brown et al. 1986), channel catfish (Mazik et al. 1994), Siberian sturgeon (Maxime et al. 1995), and Adriatic sturgeon (Di Marco et al. 1999). The return of cortisol and glucose levels to basal levels 48 h post-operation and maintenance at basal levels during blood sampling between 48-72 h post-operation permit the study of a wide variety of physiological processes and metabolic dynamics of any compound present at any desired concentration. For example, the physiological dynamics associated with the temporal stress response of sturgeon caused by the manipulation of environmental variables (Crocker and Cech 1998), or exogenous injections of stress-related hormones (Belanger et al. 2001) can be explored using the combined technique.

Subsequent to the development and validation of the combined technique, the technique has been used to study carbohydrate utilization when present in different forms (Deng et al. 2001), and in different concentrations (Gisbert et al. 2003). Tashjian

(unpublished data) has recently extended the usefulness of the combined technique by successfully applying the technique to study the kinetics of selenium absorption, distribution and excretion of L-selenomethionine, one of the most prevalent forms of selenium present in the natural food sources of the white sturgeon in the San Francisco Bay-Delta (Fan et al. 2002). The same study has provided insight into the mechanisms of acute toxicity of organic forms of selenium by demonstrating a significant decrease in urinary flow within 6 hours post-intubation, suggesting kidney failure may be the primary cause of acute toxicity and death in sturgeon intubated with elevated doses of L-selenomethionine (Tashjian, unpublished data). Additional studies investigating the kinetics of selenium absorption, distribution and excretion of L-selenomethionine when intubated at different dosage levels are in progress.

Technical Considerations

Although the use of the combined technique has help gain considerable insight into carbohydrate and selenium absorption, distribution, and excretion, there are a number of technical aspects regarding the individual components of the combined technique that must be dealt with carefully and/or improved to further refine the combined technique.

Esophageal Intubations

Many factors such as the type of carrier used, amount of material intubated, and consistency of material intubated must be carefully considered when delivering a dose of a desired compound via esophageal intubation. The type of carrier used to deliver a desired compound can greatly influence the absorption dynamics by influencing the rate of movement of the bolus through the GIT, binding with the compound being intubated (decreasing bioavailability), and competing with the intubated compound at absorption sites in the intestine, pyloric ceaca, and spiral valve of sturgeon. A recent study has demonstrated a very rapid passage time through the intestine (<3 h) and spiral valve (6-12 h) when sturgeon were intubated with a starch gel solution via esophageal intubation (Tashjian, unpublished data). Deng et al. (2000) also reported a very short passage time of a gelatin gel bolus (3-6 h) when intubated into the esophagus. In contrast, the passage time of a commercial diet through the GIT of sturgeon may be as long as 48 h (Tashjian, personal observation). Because the passage time through the GIT can have a strong influence on the absorption dynamics of a compound, the type of carrier to be used should be dependent on the objectives of the study. Simple carriers may be used to study the absorption of a compound without the interactive effects of other dietary components. More complex carriers such as commercial or purified diets (Hung et al. 1987), however, may be used as carriers to integrate the complex interactions among dietary components. The use of complex carriers may be beneficial when a more realistic understanding of the absorption and metabolic dynamics of a compound are desired (i.e. when a compound is incorporated into fish feed). The use of complex carriers may be useful to simulate a more ecologically relevant oral exposure to a compound.

The amount of intubated material in a single dose, and the diameter of the tube used, must be carefully considered in order to avoid regurgitation of an intubated bolus. Deng et al. (2000) demonstrated that sturgeon (1-2 kg) intubated with 2 g gelatin gel kg^{-1} body weight

(bw) did not regurgitate the bolus. An examination of the swim bladders (connected to the GIT in the forestomach) also demonstrated that no intubated material entered the swim bladders. However, regurgitation did occur when sturgeon were intubated with ≥4 g gelatin gel kg^{-1} bw using larger diameter tubing (O.D. 4.0 mm) during preliminary experimentation. The regurgitation could be caused by either too much intubated material or by using tubing with an outer diameter larger than the tubing used by Deng et al. (2000) (O.D. 3.2 mm). Preliminary trials with the selected intubation volume and tube diameter should be conducted before initiation of the experiments. Additional information on a variety of compound administration techniques is available in Perry and Reid (1994) and information on the bolus-injection of radiolabels for study of steady state glucose metabolism is available in West (1994).

Dorsal Aorta Cannulation

Although increased sampling frequency is desirable to obtain a more accurate understanding of the metabolic or physiological processes being studied, sampling protocols through the cannula for individual fish must be carefully chosen to minimize a number of complications. One such reported complication is the decrease in hematocrit through the sampling period in the study conducted by Deng et al. (2000). The decrease in hematocrit may be due to red blood cell mobilization and acidosis generated from anaerobic glucose catabolism, which is in turn stimulated by stress hormones (Deng et al. 2000, Soivio et al. 1972, 1975). Another explanation may be hemodilution caused by the repeated sampling of blood. Although 10% of the blood volume has been suggested as the acceptable amount that can be removed during experiment (Schultz and Hayton 1991), the amount of blood removed by Deng et al. (2000) was only 3-5% of the total blood volume. Remediations to this problem include sampling smaller volumes of blood, replacing sampled volume with an equivalent amount of blood (Axelsson and Fritsche 1994), or re-injecting the red blood cells after each sampling.

Although protocols for dorsal aorta cannulation (Deng et al. 2000) and caudal vein cannulation (Belanger et al. 2001) in white sturgeon have been developed individually, the simultaneous use of dorsal aorta and caudal vein cannulation has not been explored. Simultaneous use of both cannulation techniques may be useful when both intravascular injection and sampling is desired. The use of separate cannula for injection and sampling will prevent contamination of sampled blood. The use of a double lumen cannula may also be used to prevent sample contamination when the use of only one cannula is possible (West 1994). The choice of sampling and injection sites may influence the kinetic parameters of certain metabolic processes (West 1994,Katz 1992, Norwich 1992, Wolfe 1984), and thus the sampling and injection sites must be chosen with caution. The dynamics of compounds with a high renal extraction ratio may be especially sensitive to the chosen sampling protocol, due to a first-pass effect in the kidney (Horsberg 1994). There are also many other types of arterial and venous cannulations as described above which might also be employed with the dorsal aorta and caudal vein cannulation to expand the scope of our investigation on the biology of white sturgeon. For a detailed discussion on issues relevant to the use of cannulation techniques such as choice of cannula material and anticoagulants, physical properties of cannula, preparation of cannula, and insertion techniques, see Axelsson and Fritsche (1994).

Urinary catheterization

Urinary catheterization of white sturgeon has been the most troublesome component of the combined technique, reflected by the large variance of urinary flow rates among different studies and large variance in metabolite excretion among individuals within studies (Ng et al. 1996, Deng et al. 2000 & 2001, Gisbert et al. 2003, Tashjian unpublished data). A number of factors can be the cause of variation among and within studies including; differences in experimental conditions, physiological variation among individuals, preparation of catheters, and construction of catheters.

The 20-70% difference in urinary flow rates between the studies of Ng et al. (1996) and Deng et al. (2000) are puzzling. Although variation in sturgeon body size (nearly 2-fold difference in body size) and water temperature (7°C difference in water temperature) between the two studies may be the cause of the differences in urinary excretion rates, the difference in the urinary catheter preparation between the two studies may have been the major cause. Both investigators inserted the catheters 10 cm into the urinary ducts, but the fish used by Deng et al. (2000) were twice as big, possibly allowing water to seep into the catheters due to the shallow insertion depth of the catheters into the urinary ducts. Moreover, only 2 cm of the urinary catheters were perforated by Ng et al. (1996), while 4 cm were perforated by Deng et al. (2000), which may have caused incomplete collection of urine through the catheters in the case of Ng et al. (1996) or allowed water to be siphoned into the catheters in the case of Deng et al. (2000).

Recent experimentation on the optimum insertion depth of the urinary catheters showed that insertion of catheters between the 4^{th} and 5^{th} scutes (ca. 13 cm) anterior to the urinary duct opening resulted in blood to be present in the urine and an abnormally low volume of urine collected, possibly due to the puncturing of the urinary ducts. Insertion of the urinary catheters between the 3^{rd} and 4^{th} scutes anterior to the urinary duct opening (ca. 10.5 cm) resulted in the collection of a similar volume of urine as collected by Deng et al (2000) with no blood present. Insertion of the urinary ducts between the 2^{nd} and 3^{rd} scutes (ca. 9.5 cm) resulted in a high volume of urine collected, approximately twice of the volume collected by Deng et al. (2000, 2001). Determining the insertion depth of the urine catheters by referring to the ventral scutes rather than using a predetermined depth as used by Ng et al. (1996) and Deng et al. (2000) provides an approximate normalization of catheter insertion depth to body length.

Detailed studies on the effect of body-size and temperature on the urinary flow rate are necessary in order to correctly scale for body size and temperature effects on urinary flow rate. The influence of catheter diameter, depth of catheter insertion into urinary ducts, and degree of perforation on urinary flow rate must be determined to further optimize the catheterization technique. Further refinement of the urinary catheterization technique should also be explored to reduce variance in urine collection. A possible refinement to the technique that should be explored further includes sealing the urinary duct opening with tissue cement, which would prevent water seepage into the catheters and prevent leakage of urine not collected by the urinary catheters. Careful attention must be paid to the possible causes of variation outlined **(Table 1** at end of this article**)** in order to reduce error variation in urinary flow rate between the two catheters in each fish and among individual fish. Although difficult to confirm, accurate measurements on the urinary flow rates of undisturbed sturgeon are needed to provide a reference point to determine whether

modifications to the catheterization technique improve the collection of uncontaminated urine. For a more detailed discussion on methods for assessing kidney and urinary bladder function in fish refer to Wood and Patrick (1994).

Table 1. Troubleshooting guide when experiencing difficulties with urinary catheterization of white sturgeon.

Problem	Causes	Remediations (Troubleshooting should proceed by moving from the top to bottom of the remediation procedures)
Low urinary flow rate or no urine flow	Catheters not filled with water or air bubbles in urinary catheters	Check catheters for air bubbles. If air bubbles found, use a syringe to inject water through urinary catheters. Wait at least 24 hrs before sampling urine to allow injected water to drain out of urinary ducts.
	Height of urine collection tubes is too high, preventing a sufficient siphon of urine	Place the open tip of the catheter 2-8cm below the water-level to establish sufficient siphon (Wood and Patrick 1994).
	Height of urine collection tubes is too low causing a siphon strong enough to such urinary duct wall against catheter tip.	Place the open tip of the catheter 2-8cm below the water-level to establish sufficient siphon (Wood and Patrick 1994).
	Perforation holes in urinary catheter are blocked	Inject water into catheters with a syringe to clear blockage. Wait 24 hours before collected urine samples.
	Both urinary catheters inserted into only one urinary duct	Fish must be removed from chamber and re-operated. To prevent this problem, inject water into one urinary catheter after fish have been implanted with the urinary catheters on operating table. If water flows out of second catheter then both catheters are in the same urinary duct.[1]
	Catheterization of incorrect ducts (i.e. catheters inserted into the anus).	Remove incorrectly inserted catheterization tubes and insert into urinary ducts.[1]
	Catheters inserted too far into urinary ducts, possibly leading to puncture of catheters.	If blood is apparent in urine, remove fish from experiment. If above remediations do not solve the problem, remove fish from experiment.
High urinary flow rate	The height of urine collection tubes is too low causing a siphoning of tank water into catheters.	Place the open tip of the catheter 2-8cm below the water-level to establish sufficient siphon (Wood and Patrick 1994).
	Insertion depth of catheters is too shallow causing a siphoning of tank water into catheters	Remove fish from chambers and adjust catheter insertion depth. [1]
	High stress level in fish	Examine condition of holding chamber and fish for visible signs of stress.

[1] Because anesthesia and stress may induce a "laboratory diuresis," any manipulation of urinary catheteters during remediation procedures should be followed by a recovery period of at least 24h (Wood and Patrick 1994).

Additions to the Combined Technique

A number of additions to the combined technique should be explored to more accurately determine the absorption, distribution, metabolism, and excretion of a compound. A more accurate mass balance of a compound under study would be achieved if the excretion of the compound through the bile, feces, and gills are monitored by the combined technique. Bile duct catheterization, as established for salmon (Klonz and Smith 1968), may be attempted in the white sturgeon. Catheterization of the GIT from the anus may also be useful to recover all feces and avoid contamination of tank water so that excretion via gills may be determined. Many methods have been proposed to account for gill excretion. The use of metabolic chamber system to separate anterior and posterior parts of the body combined with the use of urinary catheterization has been developed (Smith 1929). An elevated stress response due to confinement and the potential for water contamination among chambers, however, must be explored before using this technique with white sturgeon (Wood and Patrick1994).

An alternate method without the use of a metabolic chamber system exists if the GIT and urinary ducts are fully catheterized and regurgitation of the intubated material does not occur. The alternate method is to determine gill excretion by direct water sampling (Horsberg 1994). A static system must be utilized for this type of study, and the measured compound must be present in the sampled water at detectable levels. In this type of static water study, experimental fish should be transferred through a series of static water tanks at predetermined time intervals and the water from the tanks should be sampled at the end of each time interval. If static water baths are to be used, ammonia accumulation and dissolved oxygen levels must be carefully monitored and dealt with. One complication of such studies would be the volatilization of highly volatile compounds, resulting in an underestimation of excretion rate through the gills. All additions to the combined technique must be validated to determine if results may be extrapolated to aquacultural or natural conditions.

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Primary Structures and Multiplicity of Pancreatic Hormone in Fish

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Key Words: insulin, glucagon, multiplicity, primary structure, function, immunoreactivity

Abstract

The pancreas contains several peptide hormones including insulin and glucagon which regulate nutrition metabolism and consequently growth. The primary structures and biochemical characters of these hormones are important information for the artificial synthesis of these hormones and the development of the immunoassay system. In fish, several species possess plural molecular forms of insulin and glucagons, and this phenomenon complicates the understanding of the physiological function of these hormones. The present study aimed to review the current state of knowledge concerning biochemical characters of those hormones and the systems producing multiple molecular forms.

The production systems of multiple molecular forms of insulin and glucagon are different among species. Purifying the hormones and analyzing of primary structures and gene structures elucidated this phenomenon. The production systems are classified into the following types: (1) arising from a single prohormone by proteolytic cleavage at different sites of the signal peptide (flounder insulins) or (2) C-terminus regions (ratfish insulins, barfin flounder glucagons), (3) arising from two distinct genes amplified by chromosome duplication (kaluga sturgeon insulins, paddlefish insulins and glucagons) or (4) irrespective of chromosome duplication (bonito insulins, kaluga sturgeon glucagons).

It is not clear whether there are any functional differences between each molecular form in fish. However, immunoreactive differences have been demonstrated in insulins from flounders. These differences are significant, but both molecular forms are crossreactive to the antibody. This suggests that it is difficult to measure their blood levels distinctly by immunological assays in each fish species. Thus, multiplicity of insulin and glucagon is an obstacle to develop measurement techniques for these hormones due to immunological similarities between each molecular form, but science needs to overcome this obstacle to understand the physiological function of each hormone.

Introduction

Insulin and glucagon are key hormones regulating nutrition metabolism in fish like in mammals. These hormones are contained mainly in B-cells and A-cells of the endocrine pancreas at high concentrations. Insulin is a two-chained polypeptide hormone consisting of A- and B-chains and derived from preproinsulin by proteolytic removal of a signal peptide and a peptide called C-peptide that connects A- and B-chains. Insulin secretion is induced by a feeding stimulus (Mommsen and Plisetskaya, 1991; Navarro *et al.*, 1993; Andoh and Nagasawa, 2002) and accelerates nutrition anabolism (Inui *et al.*, 1978; Plisetskaya *et al.*, 1984; Mommsen and Plisetskaya, 1991). Furthermore, insulin possesses the growth promotion function in fish. Insulin promotes sulfation uptake in gill cartilage of fish at the physiological concentration (Duan and Hirano, 1992; Duan *et al.* 1992; Plisetskaya, 1998).

Plasma insulin levels in salmonids correlated with body weight significantly and rainbow trout from fast-growing families showed significantly higher plasma insulin levels than did fish from slow-growing families (Sundby *et al.*, 1991).

Glucagon is a single polypeptide chained hormone. The proglucagon gene encodes glucagon and related peptides including glucagon-like peptide, oxyntomodulin and glicentin. These hormones are cleaved proteolytically from proglucagon. Glucagon shows a hyperglycemic effect and a depletion of liver glycogen at least in short-term experiments using fish (e.g. Ince and Thorpe, 1977; Ottolenghi *et al.*, 1989, Plisetskaya and Mommsen, 1996).

Primary structures of insulins from over 30 species (Conlon, 2001) and glucagons from over 20 species (Irwin, 2001) have been established in fish. Several of these papers showed that some fish species possessed plural molecular forms of these hormones. However, primary structure analyses of those hormones are not completely established except for limited species.

When there are plural molecular hormone forms, the following differences should be examined to understand the physiological functions, such as biological activities, immunoreactivities, and the production system (plural distinct genes or a single gene). Purifications and the establishment of primary structures of each molecular form are base information for these points.

The present study aimed to review the current state of knowledge concerning biochemical characteristics of insulin and glucagon, their systems producing multiple molecular forms and prospective way to move on to the next phase in fish.

Multiplicity of Insulin in Fish

Barfin flounder (*Verasper moseri*) possesses two molecular forms of insulin. A- and B-chains of insulin-I consist of 21 and 30 amino acid residues, respectively (**Fig.1**). Both chains of Insulin-II are 21 and 32 amino acid residues, respectively. Amino acid sequences of both insulins are completely identical to each other except for two amino acid residue extension of B-chain N-terminus of insulin-II. These characteristics and a result of Southern blot analysis suggested that these molecular forms were derived from a single preproinsulin and cleaved at different sites in the signal peptide regions (Andoh and Nagasawa, 1998a). Starry flounder (*Platichthys stellatus*) and stone flounder (*Kareius bicoloratus*) also possess two molecular forms of insulin in the same manner as in barfin flounder (Andoh and Nagasawa, 1998b, c).

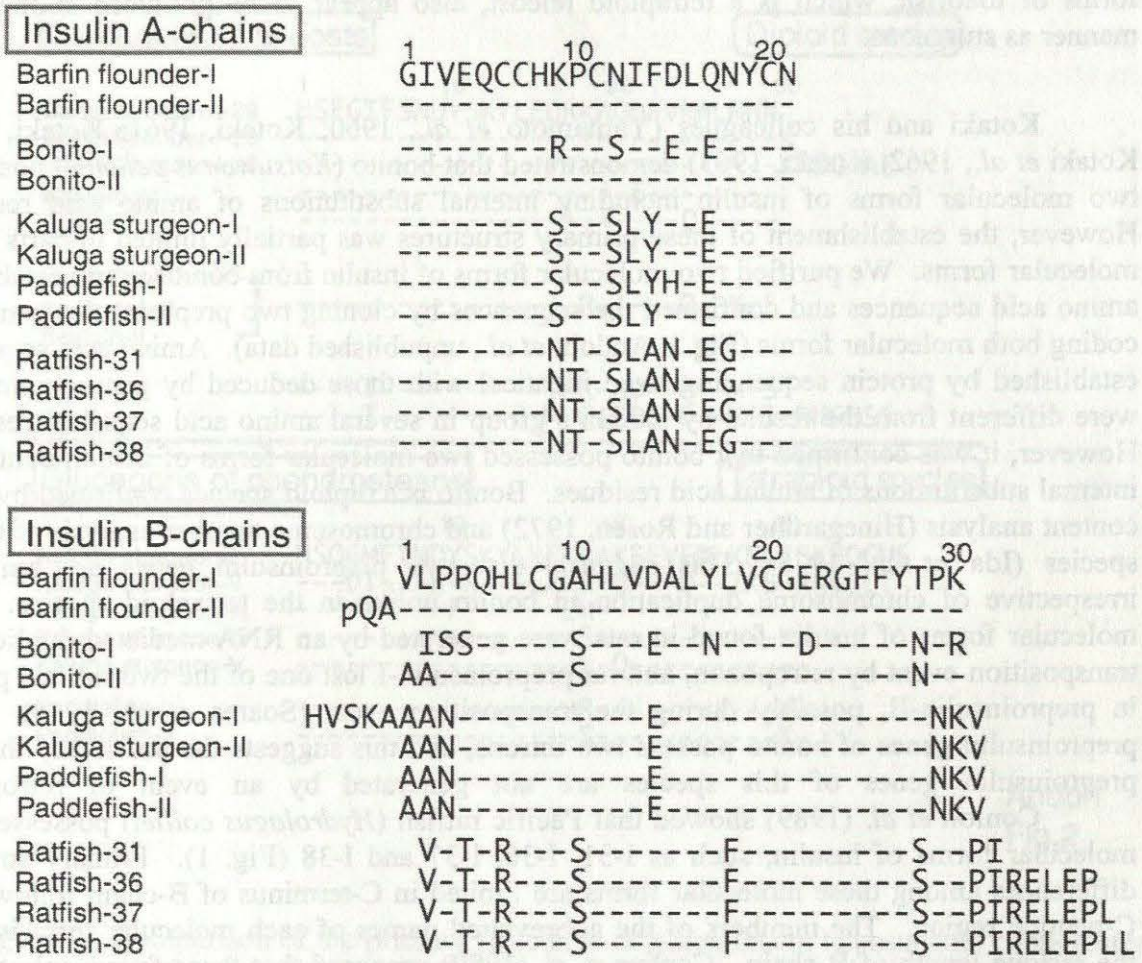

Fig. 1
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Figure 1. Comparison of the primary structures of insulin in fish. Hyphens indicate residues identical to those of barfin flounder insulin-I in other species.

Sturgeons (paddlefish, *Polyodon spathula*, Nguyen *et al.*, 1994; kaluga sturgeon, *Huso dauricus*, Andoh *et al.*, 2000) possess two molecular forms of insulin (insulin-I and –II). Those primary structures are identical to one another except for one internal amino acid residue (paddlefish insulin- I) or N-terminal five amino acid residues extension (kaluga sturgeon insulin-I) (**Fig. 1**). Two molecular forms of insulin in these chondrosteans appear to be encoded by two distinct genes amplified chromosome duplication because these species

are tetraploid species (Dingerkus and Howell, 1976; Birstein *et al.*, 1993). Two molecular forms of toadfish, which is a tetraploid teleost, also appear to be produced in the same manner as sturgeons.

Kotaki and his colleagues (Yamamoto *et al.*, 1960; Kotaki, 1961; Kotaki, 1962; Kotaki *et al.*, 1962; Kotaki, 1963) demonstrated that bonito (*Katsuwonus pelamis*) possessed two molecular forms of insulin including internal substitutions of amino acid residues. However, the establishment of these primary structures was partially limited in parts of the molecular forms. We purified two molecular forms of insulin from bonito again, established amino acid sequences and confirmed the sequences by cloning two preproinsulin genes and coding both molecular forms (**Fig.1**, Andoh, *et al.*, unpublished data). Amino acid sequences established by protein sequencing were identical with those deduced by gene cloning, but were different from the results by Kotaki's group in several amino acid sequence residues. However, it was confirmed that bonito possessed two molecular forms of insulin containing internal substitutions of amino acid residues. Bonito is a diploid species confirmed by DNA content analysis (Hinegardner and Rosen, 1972) and chromosome number analysis of related species (Ida *et al.*, 1978). This suggests that two preproinsulin genes are amplified irrespective of chromosome duplication in bonito unlike in the tetraploid species. Two molecular forms of insulin found in rats were generated by an RNA-mediated duplication-transposition event by retropozon, and rat preproinsulin-I lost one of the two introns present in preproinsulin-II, possibly during the transposition event (Soares *et al.*, 1985). Both preproinsulin genes of bonito possess two introns, and this suggests the possibility that two preproinsulin genes of this species are not generated by an event of retroposon.

Conlon *et al.* (1989) showed that Pacific ratfish (*Hydrolagus colliei*) possessed four molecular forms of insulin, such as I-31, I-36, I-37 and I-38 (Fig. 1). Primary structure differences among these molecular forms are limited in C-terminus of B-chain followed by C-peptide region. The numbers of the abbreviated names of each molecular form indicate the residue length of B-chain. Conlon *et al.* (1989) proposed that these four insulins arose from a single proinsulin by proteolytic cleavages at different sites within the C-peptide region.

Multiplicity of Glucagon in Fish

Several teleosts possess two molecular forms of glucagon (I and II) containing internal sequence residues substitutions (**Fig. 2**). Amino acid sequence similarities between glucagon-II's from sculpin (*Cottus scorpius*, Conlon *et al.*, 1987), anglerfish (*Lophius americanus*, Lund *et al.*, 1983; Nichols *et al.*, 1988), barfin flounder (Andoh, unpublished data) and tilapia (*Oreochromis niloticus*, Nguyen *et al.*, 1995) are higher than those between glucagon-I and –II in each species. Similarities between glucagon-I's are lower than glucagon-II's, but are also higher than those between glucagon-I and –II. Diploidy of these species is supported by the chromosome number analyses and DNA contents of these species or relatives (Hinegardner and Rosen, 1972). These suggest that two glucagon genes from sculpin, anglerfish and flounder were amplified irrespective of chromosome duplication and amplified before divergence of these fish species.

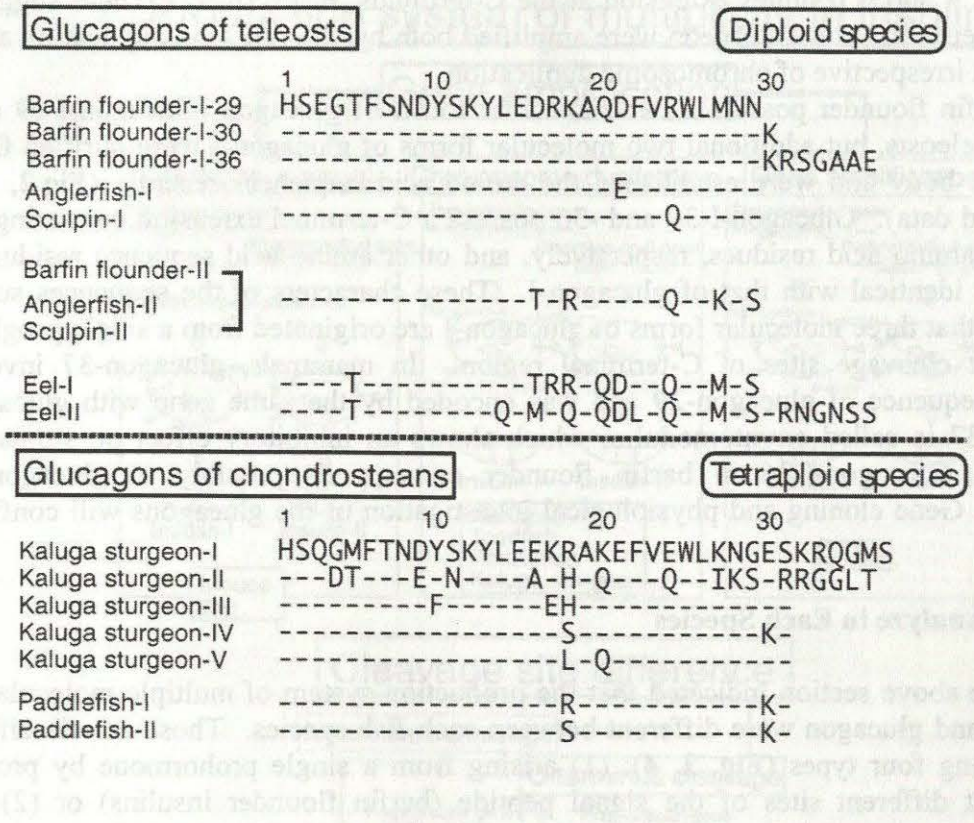

Figure 2. Comparison of the primary structures of glucagons in teleosts and chondrosteans. Teleosts and chondrosteans indicated in this figure are diploid and tetraploid species, respectively. Hyphens indicate residues identical to those of barfin flounder glucagon-I-29 or kaluga sturgeon glucagon-I in other species.

On the other hand, eel (*Anguilla anguilla*) glucagon-I gene appeared to be originated from eel glucagon-II gene irrespective of chromosome duplication and by the mechanism, which is different from those of other teleosts. Eel (Park and Grimm, 1981) is also a diploid species like the above teleosts. Eel glucagon-I shows a high degree of similarity with glucagon-II of other teleosts, while eel glucagon-II shows a low degree of similarity with glucagon-II of other teleosts. This suggests that eel glucagon-I and glucagon-II of those teleosts are more ancient and eel glucagon-II gene were generated independently by amplification of eel glucagon-I gene or its ancestral gene in eel.

Sturgeons are polyploid species. Paddlefish, a tetraploid species, possesses two molecular forms of glucagon. The number of substituted amino acid sequence is only one internal residue, suggesting a possibility that two glucagon genes were amplified by chromosome duplication. Kaluga sturgeon is also a tetraploid species like the paddlefish (Birstein *et al.*, 1993), but five molecular forms of glucagon were purified from pancreas of this species (**Fig. 2**, Andoh et al., 2000). Up until present, more than two molecular forms of glucagon have not been characterized in a singular species. Numbers of substituted amino

acid sequence between each molecular form varies from 2 to 12 residues and glucagon-I and –II possess 7 and 6 residues extension at the C-terminus, respectively. These suggest that glucagon genes in kaluga sturgeon were amplified both by chromosome duplication and by a mechanism irrespective of chromosome duplication.

Barfin flounder possesses two molecular forms of glucagon (glucagon-I-29 and -II) like other teleosts, but additional two molecular forms of glucagon-I were purified from the Brockmann body and were established the amino acid sequences recently (**Fig.2**, Andoh, unpublished data). Glucagon-I-30 and -36 possess a C-terminal extension consisting of one and seven amino acid residues, respectively, and other amino acid sequence residues were completely identical with that of glucagon-I. These characters of the sequences suggest a possibility that three molecular forms of glucagon-I are originated from a single proglucagon at different cleavage sites of C-terminal region. In mammals, glucagon-37 involved a complete sequence of glucagon-29 and was encoded by the same gene with glucagon-29. Glucagon-37 is called oxyntomodulin, which shows an inhibitory effect on stomach acid secretion. Glucagon-I-36 in barfin flounder corresponds possibly to glucagon-37 in mammals. Gene cloning and physiological investigation of the glucagons will confirm this possibility.

Points to Analyze in Each Species

The above section indicated that the production system of multiple molecular forms of insulin and glucagon were different between each fish species. Those are classified into the following four types (**Fig. 3, 4**): (1) arising from a single prohormone by proteolytic cleavage at different sites of the signal peptide (barfin flounder insulins) or (2) the C-terminus regions (ratfish insulins, barfin flounder glucagons), (3) arising from two distinct genes amplified by chromosome duplication (kaluga sturgeon insulins, toadfish insulins, paddlefish insulin and glucagons) or (4) irrespective of chromosome duplication (bonito insulins, eel glucagons, kaluga sturgeon glucagons). This complication of multiplicity is an obstacle to understanding the secretion regulation system of insulin and glucagon in fish due to the difficulty of developing a measurement system for quantification. It is necessary to simplify the analyses to solve the following four points. These points have not been analyzed until now except for some cases in a few species, but the solutions will show the way to the goal.

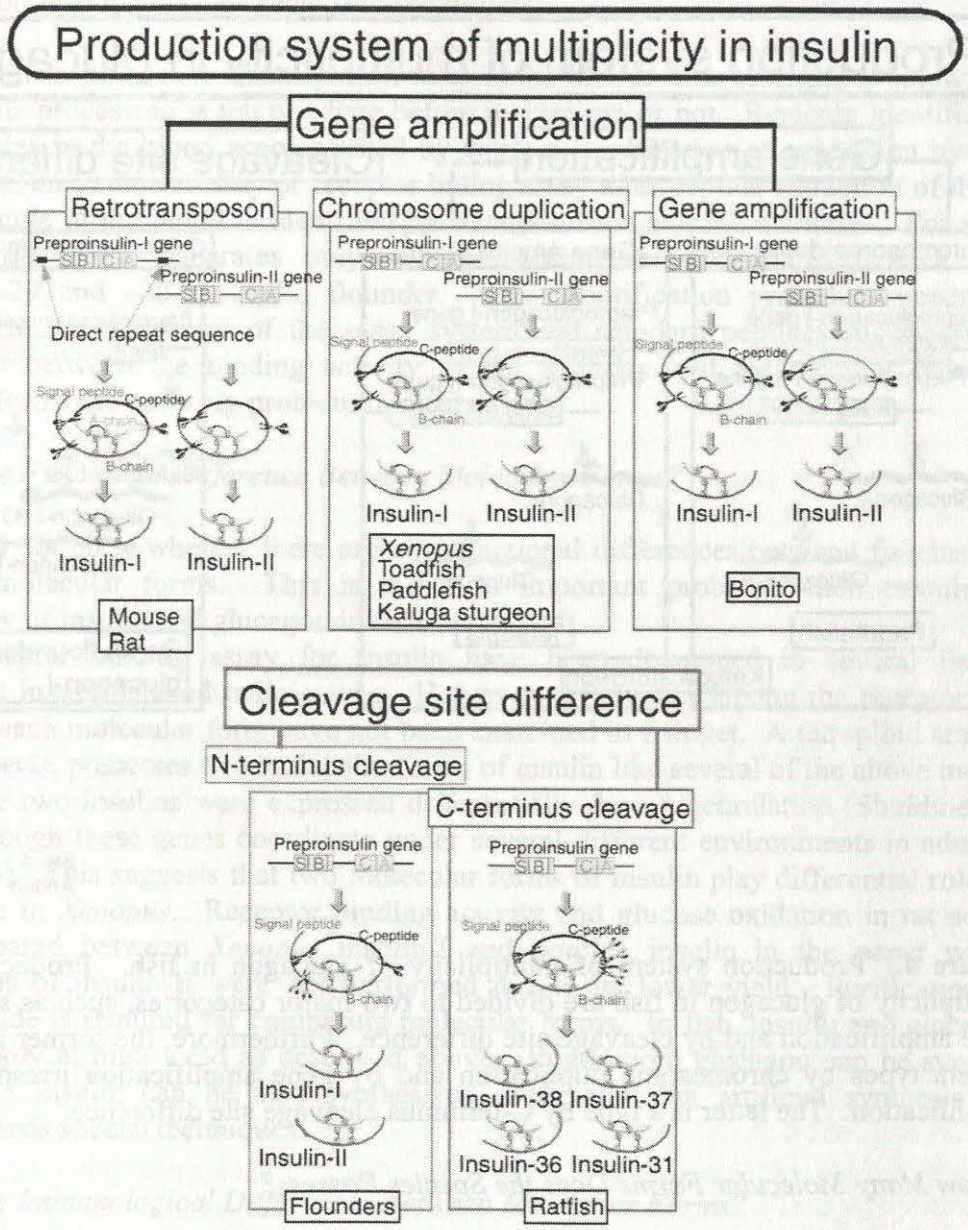

Figure 3. Production system of multiplicity of insulin in vertebrates. Production system of multiplicity of insulin in vertebrates is divided to two major categories, such as system types by gene amplification and by cleavage site difference. Furthermore, those are divided to minor categories. The former are systems delineated by by retrotransposon, by chromosome duplication and by gene amplification irrespective by chromosome duplication. The latter are divided into system types by N-terminus cleavage and by C-terminus cleavage.

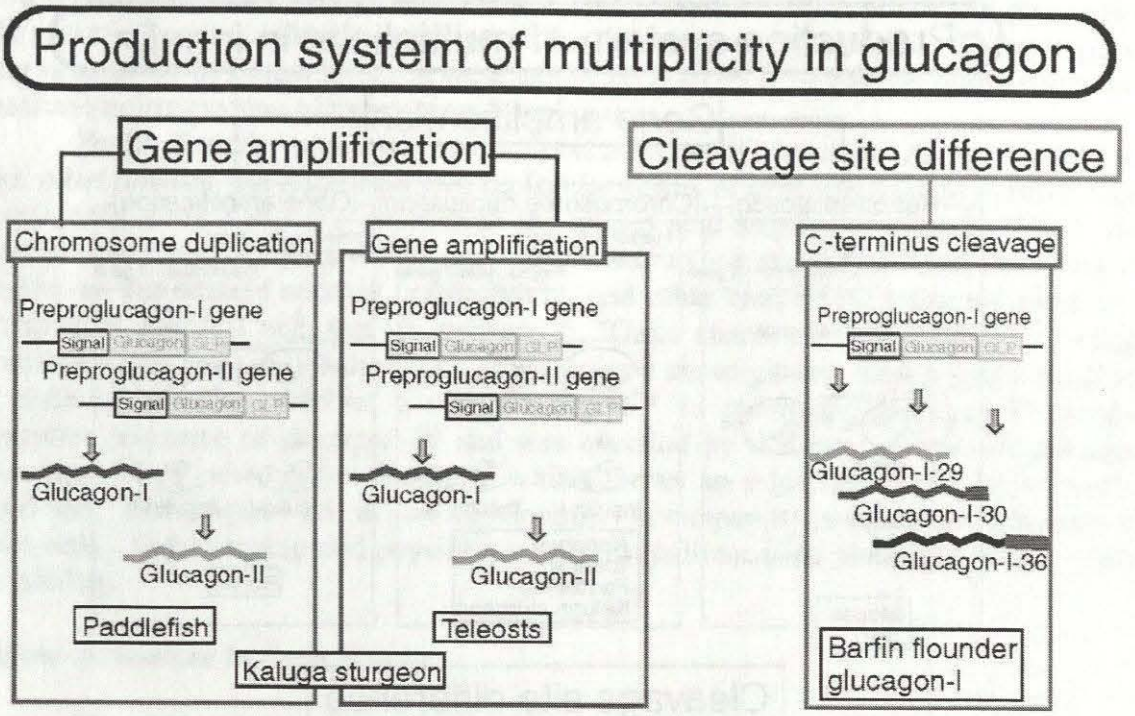

Fig. 4
Andoh

Figure 4. Production system of multiplicity of glucagon in fish. Producrion system of multiplicity of glucagon in fish are divided to two major categories, such as system types by gene amplification and by cleavage site difference. Furthermore, the former are divided into system types by chromosome duplication and by gene amplification irrespective of gene amplification. The latter is a type by C-terminus cleavage site difference.

1.How Many Molecular Forms Does the Species Possess?

Gene structure analysis and cloning are an important approach for estimating the number of molecular forms, but these methods sometimes give underestimated numbers due to the dependence on sequences of probes and primers. Therefore, purification from the Brockmann body is a better approach than gene cloning because it reflects generally on the existence ratio in the organ. The Brockmann body in fish is suitable tissue for purification of peptides unlike pancreas in other vertebrate because the Brockmann body is a pure endocrine tissue and does not contain exopancreatic enzymes. This characteristic enables one to purify peptides easily using combination of ethanol-ether precipitation or SepPak cartridge (Millipore, Milford, MA) and reverse-phase HPLC without degradation. Furthermore, biochemical characterization of each molecular form enables the determination of retention time for each molecular form in blood samples on chromatographic separation.

2. Is the Molecular Form Secreted Into the Blood?

The next point is to identify molecules in the blood. It is essential to know whether the molecule processing is intermediate before maturation or not. Realistic identification of the molecules in the blood accomplished by using a combination of separation by reverse-phase HPLC and immunoassay or receptor biding assay after peptide extraction of the blood. Each molecule in the blood is identified by retention time and the activities. For example, reverse-phase HPLC separates only a single amino acid residue difference between glucagon-I-29 and –30 in barfin flounder. This identification procedure needs a high specificity to the molecules of the assay system and standard peptides for measurement. Differences between the binding activity of the antibody and the receptor between the molecular forms are not a big problem in most cases.

3. Is There Functional Difference Between Molecular Forms?

It is not clear whether there are any functional differences between fish insulin and glucagon molecular forms. This is the most important problem when examining the multipilicity of insulin and glucagon in fish.

Receptor binding assay for insulin have been developed in several fish using mammalian insulin labeled radioisotope. However, differences among the receptor binding activity of each molecular form have not been examined in fish yet. A tetraploid amphibian, *Xenopus laevis*, possesses two molecular forms of insulin like several of the above mentioned fish. These two insulins were expressed differentially during neurulation (Shuldiner, *et al.*, 1991), although these genes coordinate under several different environments in adults (Celi *et al.*, 1994). This suggests that two molecular forms of insulin play differential roles at the larval stage in *Xenopus*. Receptor binding activity and glucose oxidation in rat adipocyte were compared between *Xenopus* insulin-I and porcine insulin in the paper while the comparisons of insulin-II were not performed due to its lower yield. Purification of the native peptide is required for comparing molecular forms. In fish, insulin and glucagon are purified easily at high yield as described above. In addition, glucagon can be synthesized artificially. Insulin can be also synthesized artificially, but artificial synthesis of this hormone needs special techniques.

4. Is There Immunological Differences Between Molecular Forms?

This point is not as important for understanding differences of physiological functions. However, an immunological detection system is the most reliable and sensitive method and does not tend to be influenced by sample components for measurement of the hormone molecule compared with receptor assay. Therefore, the immunoreactive difference between the molecular forms is sometimes an important problem to investigating secretion dynamics. Immunoreactive differences between molecular forms of insulin have been demonstrated in barfin flounder (Andoh and Nagasawa, 2002), starry flounder (Andoh and Nagasawa, 1998c) and bonito (Andoh *et al.*, unpublished data). Studies that investigate the secretion dynamics of those molecular forms needs distinct specific immunoassay systems because there may be distinct functions between molecular forms.

Practical Example in Barfin Flounder Insulin

Results of analyses of the primary structure and Southern blot showed that two molecular forms of insulin from barfin flounder arose from a single preproinsulin by proteolytic cleavage at different site of the signal peptide region (Andoh and Nagasawa, 1998a). Both insulins secreted into the blood by feeding stimulation at approximately the same ratio as that of the quantities of both insulins harbored in the Brockmann body. This indicates that the half-life of both molecular forms are the same level in the blood (Andoh and Nagasawa, 2002). Furthermore, receptor-binding activities of both insulins are completely same as the barfin flounder insulin receptor (Andoh and Matsubara, unpublished data). These suggest that there are no functional differences between the two molecular forms of insulin in barfin flounder and that two distinct immunoassay systems recognizing each molecular form are not required to measure the absolute blood level of insulin. Considering these points, we improved our assay system to create new immunoassay using B1 monobiotinylated barfin flounder insulin-I that was synthesized chemically for measurement of insulin concentration as total absolute values. Our previous assay system (Andoh and Nagasawa, 2002) used tribiotinylated barfin flounder insulin-II that was not labeled B1 amino acid residue. The B1 residue consists of an important antigenic site of insulin and only a different site between two molecular forms found in barfin flounder. Therefore, B1 labeling was expected to decrease crossreactive difference between the two insulins. The binding inhibition curves of insulin-I and –II in the previous assay system were different, but are improved in the new system (Fig. 5; Andoh, 2004). Thus, this process constructed a firm methodological basis for physiological analyses of two molecular forms of insulin in barfin flounder.

Conclusions and Perspectives

The present study reviewed primary structures, multiplicity and their production systems, and introduced a way to analyses of physiological functions of peptide hormones when plural molecular forms exist. A practical example was demonstrated for the barfin flounder. It is expected that a more comprehensive investigation of the Brockmann body of teleosts will show the existence of other molecular forms of pancreatic peptide hormones, especially glucagon-like peptide and somatostatin are also complicated due to plural molecular forms and differences among physiological functions.

Complete sequence analyses of the genomes have been finished in a lot of organism species including several fish species because of the human genome project. Recent projects are changing to focus on proteome and peptidome. Proteomics and peptidomics perform comprehensive profiling of all proteins and peptides in an organ or whole body of a species by amino acid sequence establishment.

The Brockmann body, which corresponds to islets of Langerhans in mammalian pancreas, consists of pure endocrine tissue including insulin, glucagon and other pancreatic peptide hormones in teleosts. Moreover, the Brockmann body does not contain proteases and nucleases originated from exocrine pancreatic tissue. This segregation facilitates the isolation and characterization of peptide hormones and mRNA encoding the precursors of the hormones by fewer purification steps under the inhibition of degradation unlike the mammalian pancreas. Actually, teleost is the greatest group which established primary

structure of insulin and glucagon, and the final yields of insulin and glucagon of barfin flounder possessing the Brockmann body are apparently higher than those of kaluga sturgeon possessing a mammalian type pancreas. In addition, easy purification of mRNA in the Brockmann body is more advantageous to confirm the amino acid sequences by deduction from nucleotide sequences and in quantification of transcripts. Therefore, the Brockmann body of teleosts is a suitable organ for proteomic and peptidomic analyses and the analyses will not only clarify multiple molecular forms and a possibility of existence of novel molecular forms, but also give information about the level of transcripts, translated products and the production system of each molecular form in the species. Furthermore, this analysis is a good substitute for the mammalian pancreas as a model for analysis of the insulin secretion mechanism.

Acknowledgments

The author would like to thank Prof. H. Nagasawa, the University of Tokyo, and Dr. T. Matsubara, Hokkaido National Fisheries Research Institute, Fisheries Research Agency, for their helpful suggestions and supports.

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Laser Scanning Cytometry And Tissue Microarray Assessment of Osmoregulatory Capacity in Euryhaline Teleosts

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Abstract

The size and number of mitochondria-rich chloride cells (CC) and the activity of Na^+/K^+-ATPase are good indicators of osmoregulatory capacity in teleosts. In this study we have analyzed salinity-dependent changes in CC and Na^+/K^+-ATPase using laser scanning cytometry (LSC) and tissue microarrays (TMAs) in tilapia (*Oreochromis mossambicus*) and killifish (*Fundulus heteroclitus*). Acute and gradual salinity acclimation regimens were designed and gill epithelium collected after 1, 2, and 5 weeks at fresh water, 1x seawater, or 2.4x seawater. Gill epithelial cells were dissociated and cell suspensions stained with DASPMI, followed by evaluation using a laser scanning cytometer. CC number and volume are proportional to external salinity, being lowest in FW and highest after 5 weeks in 2.4x SW. Tissue microarrays were constructed from fixed gill tissues and developed with antibody for Na^+/K^+-ATPase to visualize CCs in tissue context and compare their characteristics with isolated CCs. Na^+/K^+-ATPase per CC increases transiently after 1 week acute acclimation to 1x SW but returns to baseline within 5 weeks. Gradual acclimation to 2.4x SW also increases Na^+/K^+-ATPase per CC but this increase is stable for at least 5 weeks. CC size in tissue-context did not correlate well with external salinity because of extensive basolateral membrane infoldings. Taken together, these data demonstrate the usefulness of laser scanning cytometry and tissue microarray analysis for assessing the osmoregulatory capacity of teleosts.

A Novel Bitter Amino Acid, Pulcherrimine Isolated From Sea Urchin Ovaries: Relationship Between Maturation And Their Contents

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Key Words: sea urchin, ovary, amino acid, mature, seasonal changes

Abstract

The present study was undertaken to unders the properties and origins of the bitter substance in the sea urchin gonads and the relationship between maturation and their contents. The frequency of occurrence of bitter-tasting sea urchins collected in the sea off Iwaki, Fukushima Prefecture was examined first. The bitterness was found to be specific to mature ovaries. From the mature ovaries, a bitter substance was isolated and the structure was determined to be 4*S*-(2'-carboxy-2'*S*-hydroxy-ethylthio)-2*R*-piperidinecarboxylic acid, being a novel sulfur-containing amino acid, named pulcherrimine (Pul). Subsequently, we investigated the seasonal changes in the frequency of occurrence of mature female sea urchins and the frequency distribution of Pul contents among the mature female individuals at Iwaki, Fukushima Prefecture. Many mature individuals with high Pul-containing ovaries were found in all months examined in this area. From these findings, it was concluded that the presence of mature individuals with a high amount of Pul in all seasons is the major reason why sea urchins are hardly utilized as food in this area. In Mikuni, Fukui Prefecture, we investigated the relationship between the reproductive cycle and content of Pul in the ovaries of the sea urchin. From May to September, most of the individuals had immature gonads (stage 1), in which Pul was not detected. Many individuals were in stage 2 (growing) in October, and in stage 3 (pre-mature) in November. Pul content in the ovaries gradually increased during this period. In December and February, all the individuals were in stage 4 (mature). After the rapid decrease in GI, many individuals were in stage 5 (spent) in March. The Pul content was high in the mature and spent ovaries. These results indicate that Pul accumulates in ovaries with the progress of oogenesis.

Introduction

The sea urchin, *Hemicentrotus pulcherrimus* is widely distributed in Japanese coastal areas. It is one of the most important fishery products on the southwestern coasts of Japan, including Fukui Prefecture. However, sea urchins whose gonads taste extremely bitter are often found in the catch in the Tohoku area, including Fukushima Prefecture. Such sea urchins are not acceptable as food and have no commercial value. The present study was undertaken to examine the bitter substance in the sea urchin gonads and the relationship between maturation and their contents.

1. Isolation and structure elucidation of Pul from the sea urchin ovaries distributed in Iwaki

1.1 Frequency of occurrence of the bitter-tasting sea urchin in Iwaki

Sea urchins were collected from the sea off Iwaki, Fukushima Prefecture in March 1996 and March 1997. The numbers of specimens collected were 94 and 99, respectively. All specimens were judged to be mature, and their sex was readily distinguished by the oozed gametes (Fuji 1960). Ninety-five percent of the female individuals had bitter ovaries, while none of the male individuals had bitter testes. In March 1997, all of the urchins of which the gonads gave a bitter taste were found to be female. These results suggest that the bitterness of the sea urchin is specific to the mature ovaries (Murata *et. al.* 1998).

1.2 Isolation and structure elucidation of Pul from the sea urchin ovaries

The bitter substance was isolated from mature sea urchin ovaries using ODS column chromatography, Sephadex G-10 gel filtration, and Re-HPLC. The structure of the bitter substance was determined to be *S*-(2'-carboxy-2'*S*-hydroxyethylthio)- 2*R*-piperidinecarboxylic acid, a novel sulfur-containing amino acid by HRFABMS data, and ^{1}D and ^{2}D NMR spectral data (Murata and Sata 2000). It was named pulcherrimine (Pul) after the scientific name for *H. pulcherrimus* (Fig. 1).

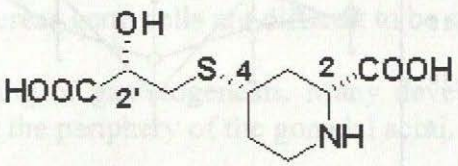

Figure 1. Pulcherrimine

The stereochemistry of Pul was determined by NOE experiments, chiral HPLC analysis and modified Mosher method (Murata and Sata 2000; Sata *et.al.* 2002).

1.3 Seasonal changes in the frequency of occurrence of mature female sea urchins and Pul content in the ovary of the sea urchin

At intervals of every three months from November 1998 to November 1999, 100 sea urchins were randomly collected from the sea off Iwaki. The sampled sea urchins were

divided into mature and immature individuals. Mature individuals were defined in this study as those with gametes, which ooze from the gonads. Immature individuals were defined as those with gametes, which do not ooze from the gonads. The sex of the mature individuals was identified from the oozed gametes (Fuji 1960). Table 1 shows the frequency of mature male, female and immature sea urchins (Murata *et. al.* 2002). In November 1998 and February 1999, all of the sea urchins examined were mature, and thus the sex of each individual was easily distinguished. In May and August 1999, the frequency of immature individuals was relatively high: 20% and 60%, respectively. This suggests that mature individuals occur in all seasons in the sea off Iwaki.

Table 1. The frequency of occurrence of mature male and female, and immature sea urchin

	Mature		Immature
	Male	Female	
November 1998	56	44	0
February 1999	57	43	0
May 1999	50	30	20
August 1999	20	21	59
November 1999	56	40	4

Seasonal changes in the GI of each sex of the sea urchin are shown in Fig. 2 (Murata *et. al.* 2002). The GI values showed a large variation among the mature specimens in each season. The fact indicates that the maturation process of the sea urchins might vary among individuals. The mean GI values of gonads were observed to decrease significantly during the period between February and May 1999, being at the lowest values in May 1999, and to increase thereafter. This change in the GI suggests that the major spawning season is in the period from February to May.

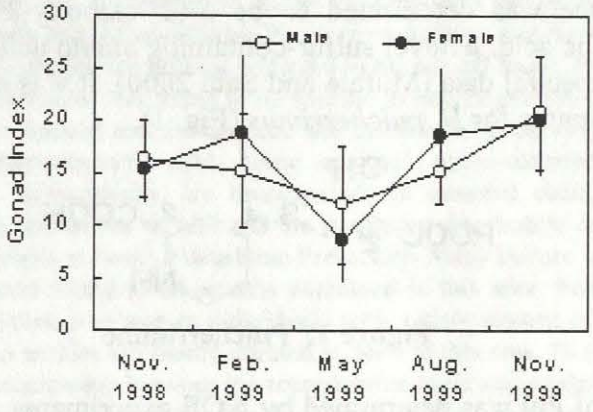

Figure 2. Changes in GI of mature male and female individuals

From the variation of the individual GI values and the presence of mature individuals in all seasons, the reproductive cycle of this species of sea urchin is unclear, and the occurrence of the mature stage extends over a long term, at least, in this sea area.

Twenty specimens were randomly selected from each of mature ovaries and testes and analyzed for Pul content (Murata *et. al.* 2001) in each month. No Pul was detected in

mature testes, and they had no bitter taste. Changes in the mean Pul content in ovaries are shown in Fig.3. Many mature females with high Pul-containing ovaries were found in all seasons. Pul levels of ovaries were the highest in February. However, mean Pul content of ovaries were more than 0.8 mg/100g in all seasons. From these findings, it was concluded that the presence Pul in mature female ovaries is the major reason that sea urchins are hardly utilized for food in the Iwaki area.

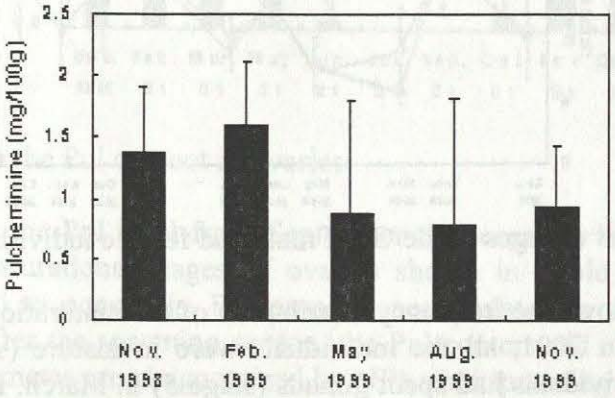

Figure 3. Seasonal changes in the mean Pul content in ovaries

2. Relationship between the reproductive cycle and the content of Pul in *H. pulcherrimus* ovaries

At intervals of one to three months from December 2000 to December 2001, 10-30 *H. pulcherrimus* were collected off Mikuni, Fukui Prefecture. The gonadal maturity of each individual was classified into the following stages initially proposed by Fuji (1960) and then modified by Unuma (2002).

Stage 1 (Recovering): Immature gonads before gametogenesis. Gonadal acini are filled with nutritive phagocytes (NPs). In females, a few young oocytes are present at the periphery of the acini. In males, haematoxylin-stained speckles, residue from phagocytized spermatozoa, are often present in NPs, whereas germ cells are difficult to be seen.

Stage 2 (Growing): Beginning of gametogenesis. Many developing oocytes or clusters of spermatogonia are present at the periphery of the gonadal acini.

Stage 3 (Pre-mature): Middle of gametogenesis. In the center of the gonadal lumina, NPs are replaced with ripe ova or spermatozoa.

Stage 4 (Mature): Fully-mature gonads at the end of gametogenesis. The gonadal lumina are filled with ripe ova or spermatozoa.

Stage 5 (Spent): After spawning, the gonadal lumina have numerous empty spaces and a few residual ova or residual spermatozoa.

Changes in the GI of each sex are shown in Fig. 4. In 2001, the mean GI values of male and female individuals rapidly decreased from February to March, and then tended to increase.

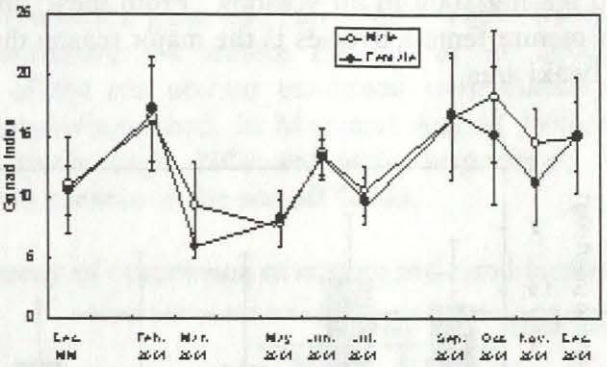

Figure 4. Seasonal changes in the GI of male and female individuals

Table 2 shows the frequency distribution of the maturational stages of gonads in each month sampled. In 2001, all the individuals were in mature (stage 4) in February, while greater part of individuals had spent gonads (stage 5) in March. From May to September, all the individuals with one exception in May had immature gonads (stage 1).

Table 2. Frequency distribution of the maturational stages of gonads

Date	Total Number	Maturational stages of gonads									
		Male					Female				
		1	2	3	4	5	1	2	3	4	5
Dec. 20, MM	13				11					2	
Feb. 16, 01	25				10					15	
Mar. 27, 01	23				4	11				1	7
May 30, 01	30	17				1	12				
Jun. 26, 01	20	8					12				
Jul. 25, 01	25	13					12				
Sep. 18, 01	10	4					6				
Oct. 25, 01	24	1	9	5				9			
Nov. 19, 01	19			6	1			5	7		
Dec. 25, 01	20				12					8	

Many individuals were in stage 2 (growing) in October and in stage 3 (pre-mature) in November. All the individuals were mature (stage 4) in December. These results in the maturational stages, taken together with the changes in GI, suggest that *H. pulcherrimus* in Mikuni initiates gametogenesis around October and spawns around February to March.

Changes in the Pul content in ovaries are shown in Fig. 5. The mean Pul level steadily decreased from December 2000 (2.6 mg/100g) to July 2001 (0 mg/100g), and kept this value until September 2001. The values started to increase from October 2001 and reached the maximum in December 2001 (3.5 mg/100g).

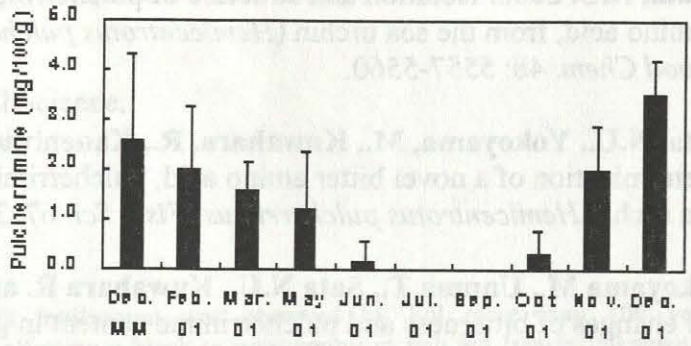

Figure 5. Changes in the Pul content in ovaries

The increase in the Pul level from September to December was in accordance with the progress of the maturational stages of ovaries shown in Table 2, indicating that Pul accumulation is related to oogenesis. Pul possibly accumulates in the oocyte or ovum as oogenesis proceeds. After the spawning season, the Pul content did not decrease rapidly. In sea urchins, residual gametes are phagocytized by NPs after spawning (Masuda & Dan 1977; Tominaga & Takashima 1987). Pul detected in the immature ovaries in May and June could be derived from the phagocytized residual ova. Probably, this Pul was degraded as the phagocytized ova gradually de-generated.

In conclusion, Pul accumulates in the ovary of *H. pulcherrimus* as oogenesis proceeds and gradually disappears after spawning.

Acknowledgments

The authors appreciate the valuable advice from emeritus Prof. Morihiko Sakaguchi at Kyoto University. The author is greatly obliged to Prof. Shiro Konagaya at Kokugakuin Tochigi Junior College, for critical reading of the manuscript. The author is also grateful to Prof. Michio Shigei at Kyoto Institute of Technology, for the species identification.

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Blocks to Polyspermy in Fish: A Brief Review

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Abstract

Although fertilization was observed by Fol more than 100 years ago, mechanisms for effecting a block to polyspermy in fish are largely unknown. In this review, the recent studies of polyspermy block mechanisms in teleost fish eggs will be presented, including morphological, biochemical, and molecular results.

Introduction

In the animal world, to survive and leave offspring, each species has evolved different mechanisms. In mammals, the upper reaches of the female's oviduct, the infundibulum, is the site of fertilization, and a special site to implant the embryo has evolved, the uterus, where subsequent development /nutritional support takes place. After implantation in the uterus the mammalian fetus develops the respiratory, excretory and nutritive supply line directly to the mother, the placenta. Another special organ is the mammary gland to feed the newborn on mammary secretions. Compared with other species, mammals are able to give birth to a small number of offspring at one time because their guardian system for embryo growth and parental care including lactation provides increased safety and augments the likelihood of continued development for the most vulnerable stages (Jameson, 1988).

In contrast to mammals, oviparous fish have developed a system to spawn a huge number of eggs and to fertilize them at the same time. Before spawning, during oogenesis in the female, the vitellogenin and choriogenin are synthesized in the liver under the influence of estrogen and accumulate in the oocyte as the yolk to serve as nutrition for the embryo, and in the egg envelope (chorion) to protect the embryo from the chemical, physical and biological pressure in the environment (Yamagami et al., 1994). While the parents produce a huge number of fertilized eggs at one time, the number of embryo to survive to maturity is very small. Under natural conditions, fertilization must be completed between one maternal gamete (oocyte, egg) *vs.* one paternal gamete (sperm). It is fatal if more than one sperm fuses with the oocyte, and most embryos die at early stage. During fertilization, a huge number of sperm compete with each other to fertilize an oocyte. Among the fish except for the sharks and chimaeras, only a single sperm penetrates into the cytoplasm of the oocyte. In the case of sharks and chimaeras, more than one sperm normally enters the oocyte, while only one sperm nucleus fuses with the oocyte nucleus, although the mechanisms controlling these physiological polyspermic fish eggs is unclear. For monospermic eggs, mechanisms to prevent supernumerary sperm penetration into the oocytes, called "the block to polyspermy or polyspermy block," must exist, and to date a large part of these mechanisms remains unclear. In this review, the current understanding of major strategies employed by the fish egg to block polyspermy will be discussed.

Comparison between the mammalian fertilization and oviparous fish fertilization

Historically, many investigators reported the importance of an intact egg envelope (chorion, vitelline envelope, zona pellucida, zona radiata) to maintain monospermic fertilization in the fish egg. When the egg envelope is removed from the unfertilized egg followed by insemination, the egg always becomes polyspermic (Kanoh and Yamamoto, 1957; Yanagimachi, 1957; Sakai, 1961; Iwamatsu, 1983; Iwamatsu and Ohta, 1978; Ginzburg, 1961; Gamo et al., 1960). In oviparous fish eggs, there is the unique structure called the micropyle on the surface of the egg envelope where the sperm can only enter to go through and attach to the oocyte plasma membrane; this structure does not exist in the mammalian egg.

Figure 1 shows a comparison of the fertilization process between mammals and oviparous fish. In mammals, to succeed in fertilization, sperm must first bind to the egg envelope, the zona pellucida (**Fig.1a**). The egg envelope of mammals consists of at least three major glycoproteins, ZPA, ZPB and ZPC. Since 1985 when Florman and Wassarman identified ZPC as the sperm receptor, specifically its O-linked carbohydrate chains, many investigators have studied the molecular mechanisms of sperm-egg interaction in mammals.

After the sperm bind to ZPC, a component of the sperm acrosome, located at the sperm head, called "acrosin," is released to digest the egg envelope (zona pellucida) glycoproteins to make it possible for fusion between the sperm plasma membrane and oocyte plasma membrane (acrosomal reaction, **Fig.1b**). In 1988, Bleil et al identified ZPA is the secondary sperm receptor for sperm during the fertilization process in mice to maintain the binding of acrosome-reacted sperm to the egg ZP during penetration. The attachment of the sperm plasma membrane to the oocyte plasma membrane induces the Ca^{2+} wave in the egg to the opposite site from the sperm-egg attachment site, following the release the contents of the egg cortical granules (cortical reaction). During the cortical reaction, the contents of the cortical granules interact with the egg envelope (zona reaction) and no more sperm can penetrate into the oocyte.

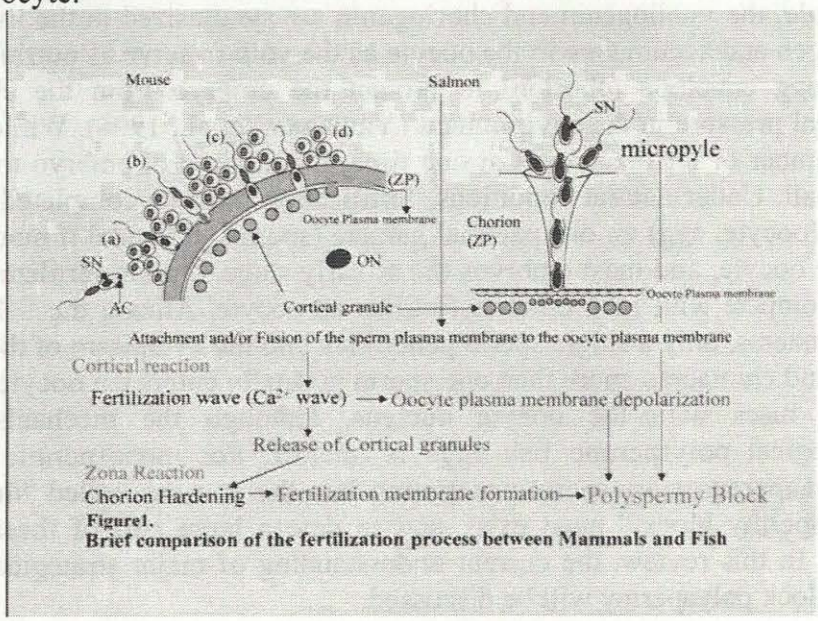

Figure 1. Brief comparison of fertilization process between mammals and fish

SN; sperm nucleus, AC; acrosome, ON; oocyte nucleus, ZP; egg envelope (zona pellucida, chorion)

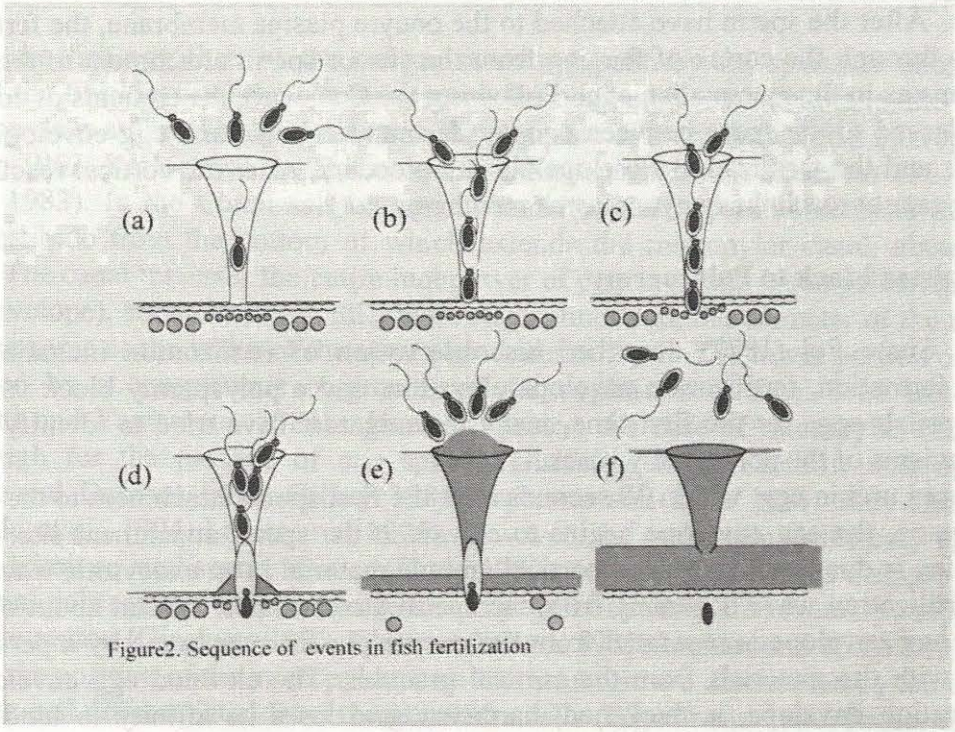

Figure2. Sequence of events in fish fertilization

Figure 2 shows the summary of the mechanisms of polyspermy block in fish egg:

(1) The diameter of the inner aperture of the micropyle restricts the number of sperm entering the micropyle. (**Fig.2a**)

(2) The fertilization cone prevents excess spermatozoa from attaching to the oocyte plasma membrane and/or from entering into the perivitelline space. (**Fig.2b, 2c**)

(3) Cortical granule lectins immobilize the excess sperm in the micropylar vestibule. (**Fig.2d, 2e**)

(4) The perivitelline fluid containing cortical granule lectins as the sperm agglutinin flows out along with excess spermatozoa through the micropylar aperture, caused by the increase of osmotic pressure. (**Fig.2e**)

(5) Perivitelline fluid containing cortical granule lectin through the micropyle interacts with the outside of the fibril layer to eliminate sperm guidance and attractant to the micropyle. (**Fig.2f**)

(6) Hardening of the chorion by alveolin and transglutaminase following inner layer chorion shrinkage decreases the diameter of the micropyle or entirely closes the micropyle resulting in shutting out of supernumerary sperm. (**Fig.2f**)

Compared with the process of mammalian fertilization, fish oocytes have the micropyle that consists of a vestibule and a micropylar canal, which is generally funnel-shaped (see detail below). The sperm can attach to the oocyte plasma membrane only through the micropyle, therefore for fish sperm, it is not necessary to bind the egg envelope (chorion). This may be one of the reasons why most fish sperm do not have an acrosome. In

some fishes, the existence of an acrosin-like protein has been reported but its function still remains unknown.

After the sperm have attached to the oocyte plasma membrane, the fertilization wave moves through the cortex of the egg from the site of sperm attachment to the opposite site, the same as in the mammalian egg. Following the Ca^{2+} wave the contents of cortical granules are released to the space between oocyte plasma membrane and egg envelope (perivitelline space), and the fertilization envelope elevation occurs. After the cortical reaction, additional sperm cannot reach the oocyte plasma membrane.

Two-phase block to Polyspermy

Since Fol (1877) described his observation of fertilization including perivitelline space formation, fertilization envelope elevation, and a polyspermy block using sea urchin and starfish eggs for the first time, many investigators have tried to identify the molecular mechanisms of the polyspermy block.

In the sea urchin egg, within 30 seconds after the first sperm attachment to the oocyte plasma membrane, the egg envelope begins to elevate at the sperm attachment site. This envelope elevation is due to the release of cortical granule material in an exocytotic wave, triggered by the fertilization wave occurring from the sperm attached site. Within about one minute, the entire egg envelope is separated from the oocyte plasma membrane by a perivitelline space filled with the materials from the cortical granules. The elevated egg envelope, called the fertilization envelope, is thickened, hardened, and loses its affinity to bind sperm. These changes are caused by the interaction between the egg envelope and the materials secreted from the cortical granules (Fol 1877, Just 1919, Epel et al., 1977).

In 1952, Rothschild and Swann observed that the prevention of polyspermy is complete within one minute after sperm penetration and before the hardening of the fertilization envelope itself. Then Rothschild and Swann elaborated their hypothesis to a two-phase block to polyspermy using sea urchin fertilization model. According to their model, in the first 1~2 seconds after the first sperm attaches to the oocyte plasma membrane, certain invisible changes spread in the surface of the oocyte rendering it less permeable to other spermatozoa (rapid partial block phase). The second phase is slower and corresponds to changes visible in the cortex: after completion the egg surface is completely impermeable to spermatozoa (the full block phase). This hypothesis has been widely cited to explain the entire process of block to polyspermy.

The mechanisms of polyspermy block in the fish fertilization

The mechanism of the polyspermy block in fish eggs consist of at least four steps; (1) a polyspermy block related to changes in the structure of the micropyle to reduce the number of sperm reaching the oocyte plasma membrane, (2) formation of a fertilization cone derived from the sperm and oocyte plasma membranes to plug the inner opening of the micropyle (3) interaction between released cortical granule lectins and unfertilized supernumerary sperm in the micropylar vestibule, and (4) modification of the egg envelope by the contents released from the cortical granules (zona reaction including egg envelope hardening). This review will be focused on the events occurring in the micropylar region during the fertilization process related to the mechanisms of polyspermy block in the fish egg.

Step 1. Polyspermy block related to the structure of micropyle: The micropyle

In the fish egg there is the unique structural pathway for sperm to enter from the surface of the fibril layer of the oocyte to reach the oocyte plasma membrane, the micropyle. The micropyle is a funnel-shaped structure that consists of a vestibule and a micropylar canal (Ginzburg 1963, Kudo 1980, 1982, Laale 1980, Kobayashi and Yamamoto 1981, Hart and Donovan 1983). In the Chum salmon egg, the micropylar apparatus consists of a funnel-shaped vestibule from the bottom of which extends the micropylar canal, about 5 μm in diameter. The canal traverses the entire inner layer of egg envelope (zona pellucida interna of the egg envelope), about 30 μm in thickness. At its inner end, the diameter of the micropylar canal is the same as the width of the sperm head, about 2 μm. The lower portion of the canal is occupied by a conspicuous outgrowth of the ooplasmic surface, 3-10 μm in length (Kobayashi and Yamamoto, 1981). In most species, the base of micropylar canal is only wide enough for the passage of one sperm (Ginzburg 1972, Kuchow and Scott 1977, Brummett and Dumont 1979, Gilkey 1981, Iwamatu and Ohta 1981, Hart and Donovan 1983, Kudo et al., 1994, Leiher et al 1995), however, the micropyle in the carp egg is wide enough to admit several sperm at once (Kudo 1980). In most fish there is a single micropyle at the animal pole region, while in sturgeon and paddlefish there are several micropyles at the animal pole region (Ginzburg 1972, Cherr and Clark 1982, Linhart and Kudo 1997). During oogenesis the position of the micropyles(s) and its structure are determined by the micropylar cell or plug cell differentiated from the granulosa cells (Eigenmann 1890, Laale, 1980, Riehl 1978). It is obvious that the function of the micropyle during fish fertilization is to restrict the number of sperm to one or several from the numerous sperm trying to enter and attach the oocyte plasma membrane. In 1961, Sakai et al. reported that in Oryzias latipes, eggs with the envelope removed become polyspermic. This observation indicated the importance of the micropyle and egg envelope to the polyspermy block. Following the first sperm attachment to the oocyte plasma membrane through the micropyle, the egg surface responds to this stimuli and the Ca^{2+} wave moves along the egg cortex from the sperm entry site to the opposite side. Following this Ca^{2+} wave, the contents of cortical granule are released (cortical reaction) into the space between oocyte plasma membrane and egg envelope (perivitelline space formation).

The lack of the electrical polyspermy block

As described above, the process to prevent the supernumerary sperm into the oocyte consist of two different time lagged steps, 1) the rapid partial block phase, and 2) the full block phase. In some fish it seems that the fertilization wave may not be caused by the rapid partial block phase. In 1980, Nuccitelli et al. reported that the fertilization potential of Oryzias latipes has only a small positive going phase and voltage clamping the egg membrane at potentials between –80 and +48 mV does not prevent fertilization. In trout eggs, Ginzburg found that the trout egg remains fertile for a period of time after insemination in water (40 seconds at 3 °C and 8.9 °C), and then after 60 seconds fertility decreases sharply and is lost completely within 110-120 seconds. A cytological investigation indicated that the cortical secretion begins only after 60 seconds in water, i.e., 30-40 seconds after the activation wave appears. This means that the trout egg retains its original fertilization

potential until the contents of the cortical granules begin to discharge into the perivitelline space (Ginzburg, 1963a,b, 1972, Kusa, 1950, Devillers et al., 1954, Zotin, 1954). It is not clear whether all fish lack an electrical polyspermy block; other species without an electrical block are the salamander (Charbonneau et al 1983) and some mammals (Miyazaki and Igusa, 1981, 1982, Igusa et al., 1983, Jaffe et al., 1983, McCulloh et a., 1983, Braden 1954). The investigations in fish suggest that the function of the narrow channel for sperm entry, the micropyle, the interaction between the cortical granule contents and sperm during the cortical reaction, and the modification of the egg envelope by cortical granule contents are essential for the block to polyspermy.

Step 2. Formation of the fertilization cone: Fertilization cone

In 1981 Kobayashi et al. reported that in the Chum salmon egg the first contact of the ooplasmic surface with a fertilizing sperm occurred at the apex of the outgrowth in the micropylar canal. The surface of the outgrowth undergoes changes immediately after penetration by a fertilizing spermatozoon that prevents supernumerary spermatozoa from penetrating the ooplasm. Similar outgrowths from the egg ooplasm into the micropylar canal were observed in the sturgeon egg (Ginzburg 1959) and in the carp egg (Kudo 1980). In the *Fundlus heteroclitus* egg, a short cytoplasmic projection at the animal pole of the unfertilized egg was observed by Brummet and Dumont (1979) and sperm-oocyte plasma membrane fusion occurred at the apex of the projection inserted into the micropylar canal. In the zebrafish (*Brachydanio*) egg, a similar small projection in the micropyle was observed, while in the case of the medaka egg, a similar structure as the outgrowth in chum salmon or as a small projection in *Fundrus* and carp was not observed in the micropylar canal before or after the gamete fusion (Iwamatsu and Ohta 1978, 1981). This outgrowth and /or small projection from the egg cytoplasm into the micropylar canal has been termed the "fertilization cone" (Kudo 1980, Kobayashi and Yamamoto 1981, Hart and Donovan, 1983, Wolenski and Hart, 1987). It has also been reported that the fertilization cone is an actomyosin complex of the fused sperm and oocyte plasma membranes on (Gilkey et al., 1978, Hart et al., 1992, Abraham et al., 1993a,b) located between the ooplasm and the egg envelope to prevent a huge number of sperm access to the perivitelline space. It is obvious that the function of the fertilization cone in the block to polyspermy is to plug the inner opening of micropylar vestibule to block the supernumerary sperm from attaching and/or to penetrating into the space between egg envelope and oocyte plasma membrane.

Step 3. The interaction between released cortical granule lectins and supernumerary sperm in the micropylar vestibule

The cortical granules are specialized Golgi-derived secretory granules located just below the plasma membrane of mature unfertilized egg. As described above, after the first (fertilizing) sperm attaches to the oocyte plasmamembrane, the fertilization wave moves through the cortex of the oocyte from the site of sperm attachment to the opposite side of the oocyte. Following this fertilization wave, the contents of cortical granule are released into the perivitelline space. It is known that cortical granules contents contribute to the block to polyspermy. In this section, the function of the cortical granule lectin in the polyspermy

block during fish egg fertilization will be discussed. The function of other contents' contribution to the polyspermy block will be discussed in Step 4.

Egg Lectin

Since the first discovery of the contribution of an egg lectin in the polyspermy block in the anuran amphibian *Xenopus laevis* (Wyrick et al., 1974), our laboratory has focused on the function of fish egg lectin during fertilization. Many researchers reported the existence of lectin in the fish egg, but its function was unknown. We chose the Chinook salmon as a model system to study the function of the egg lectin using biochemical and molecular biological methods because of its advantage in obtaining large amounts of biological materials and its economic importance in California. The eggs and milt were collected at the Nimbus State Hatchery (Department of Fish & Game, Nimbus, California) and transferred immediately to the lab in nearby Davis, California, at 4 °C. The egg lectin was purified with rhamnose-coupled affinity column chromatography and HPLC with ion exchange column and/or C4 reverse phase column chromatography. The salmon egg lectin (SEL) was found to consist of three subunits, SEL24K, SEL26Ka, and SEL26Kb, named by their molecular weights determined by SDS-PAGE. Each subunit of SEL was characterized using biochemical methods and cloned from a Chinook salmon ovary cDNA library (Murata et al., unpublished data).

Sperm agglutination and sperm immobilization activity of SEL24K

Sperm agglutination activity was measured by mixing 1/100 diluted milt and purified subunits of SEL that had been dialyzed against 10mM HEPES buffer pH 7.5. The sperm mixed with SEL24K agglutinated to each other immediately, and this activity was inhibited by adding EDTA (10 mM) but not excess rhamnose. Adding excess Ca2+ or Mg2+ blocked the inhibitory effect of sperm agglutination by EDTA. Sperm motility was also examined, and we found that SEL24K has sperm immobilizing activity, while SEL26K did not possess any sperm immobilizing activity. The sperm immobilizing activity by SEL24K was not inhibited by excess rhamnose. Therefore, SEL24K has three different activities: 1) carbohydrate binding activity, 2) sperm agglutination activity, and 3) sperm immobilizing activity. From our results, SEL24K should have at least two different domains, one is carbohydrate-binding domain and another is sperm binding domain (Murata et al., unpublished data).

The localization of the SEL in unfertilized and inseminated eggs

The localization of SEL was determined for the unfertilized and fertilized egg using immunohistochemical and immunocytochemical methods. In unfertilized eggs SEL was located not only in the large cortical granules but also in the small cortical granules just below the micropylar canal (Kobayashi et al., unpublished observations). The localization of the SEL ligand was also identified using purified SEL labeled with green fluorescence as a probe, and the signal was detected at just outside of the fibril layer of the outer layer of the vitelline envelope. Within one minute after insemination, SEL was located in the perivitelline space, the edge of the inner layer of the vitelline envelope, and in the micropylar vestibule in

which a large number of sperm were observed (Kobayashi et al., unpublished observations). This data was supported by the detection of SEL in perivitelline fluid analyzed by Western blot. Two minutes after insemination the signal was still detected in the micropylar vestibule while the supernumerary sperm had disappeared from the micropylar aperture. Judging from these data, SEL was released from the cortical granules triggered by the fertilization wave into the perivitelline space, and filled the perivitelline space and micropylar vestibule. At this point SEL interacts with sperm to agglutinate and immobilize them, to push out the excess sperm from the micropylar vestibule as a fertilization plug, and to modify the fibril layer to hide the groove to the micropyle at the surface of fibril layer. The result is that sperm cannot find the micropyle in the fertilized egg. I propose that SEL is the main molecule to contribute to the polyspermy block during the period from the beginning of the cortical reaction to the completion of the modification of egg envelope including egg envelope hardening. We have cloned medaka egg lectin subunits from a medaka ovary cDNA library (Murata et al., unpublished data).

In 1961 and 1972, Ginzburg reported the existence of a substance having sperm agglutination activity and sperm immobilizing activity in the perivitelline fluid of trout eggs. A similar observation was done in Chum salmon eggs by Janna and Yamamoto (1984) and in medaka eggs (Iwamatsu et al., 1997). These observations are also support my hypothesis. These functionally essential molecules may be conserved in many species. In 1980, in carp fertilized eggs, Kudo reported that in the perivitelline space near the canal, a few spermatozoa were frequently found and at about 6 seconds after immersion in fresh water to activate the eggs, agglutination of spermatozoa was usually observed in or near the micropylar vestibule. This observation suggested that in carp eggs it was possible that more than one sperm could enter into the inside of oocyte through micropyle but materials like SEL from cortical granules trapped them so that they could not penetrate the oocyte. This observation also strongly supported to our hypothesis.

Fertilization plug

Many investigators have observed that the slow burst of the perivitelline fluid through micropylar canal pushes excess sperm out of the micropyle and often forms a ball-like plug called the "fertilization plug" at the outside of the micropylar vestibule, composed of a colloidal substance originating from the cortical granules (Yamamoto 1952a,b, 1953, Sakai 1961, Kobayashi and Yamamoto 1981, Hart and Donovan 1983, Kudo and Sato 1985, Iwamatu et al., 1991, 1997). As described above, the fertilization plug is different from the fertilization cone. The fertilization plug is formed after the micropylar vestibule is plugged by the fertilization cone. This colloidal substance may include the cortical granule lectin to agglutinate and immobilize the excess sperm in the micropylar vestibule.

Step 4. Modification of the egg envelope by cortical granules contents (zona reaction including egg envelope hardening): The mechanisms of chorion hardening

During the fertilization process in fish, following gamete fusion, the morphological and biochemical changes in the extracellular matrix, the egg envelope (vitelline envelope, chorion) to the fertilization envelope, is the most dynamic transformation. According to the review of Yamamoto T., (1961), the first description of the characteristic differences between

unfertilized egg envelopes and activated egg envelopes using *Onchorhynchus keta* was done by Aoki (1941). In 1958, Zotin found a hardening enzyme in the perivitelline space of salmon eggs that is secreted from the cortical layer but not from cortical alveoli (granules). The hardening process by this hardening enzyme is blocked by oxygen deficiency. In 1960, Ohtsuka described that the hardening is due to an oxidation of the SH-groups and aldehyde produced by oxidation of alpha-glycol groups. In medaka, the egg envelope consists of three major subunits, ZI-1 (76 kDa), -2 (74kDa) and -3 (49kDa), and their origin is the liver of spawning females but not the ovary (Murata et al., 1991, 1994, 1995, 1997). In 1991, Masuda et al. demonstrated that the solubility of the proteins in 1N NaOH decreases to 20% of that of proteins of unhardened egg envelopes 60 min after ionophore activation of eggs. During the early hardening process, ZI-1 and -2 undergo limited hydrolyzed to produce 58-61 kDa proteins which are then polymerized with ZI-3 to form insoluble higher molecule weight complexes (135 kDa) (Masuda et al., 1991, 1992, Iuchi et al., 1995).

In this review, the function of two different enzymes involved in the chorion hardening will be discussed.

Alveolin

In 2000, Shibata et al. discovered that a metalloproteinase in the cortical granules triggered chorion hardening during the cortical reaction in the medaka egg. They purified it as a 21.5kDa protein from the exudates of the cortical alveoli (granules) and obtained the cDNA sequence for this metalloproteinase. From the predicted amino acid sequence, this metalloproteinase was identified as a member of astasin metalloproteinase family and was named "alveolin" after its origin, the cortical alveoli. Alveolin was found to process ZI-1 and -2 to 61-62 kDa intermediates, which are then cross-linked with ZI-3 by transglutaminase within the egg envelope itself.

Transglutaminase

The formation of gultamyl-lysine isopeptide crosslinks was suggested to be a cause of chorion hardening in trout eggs by Hargenmier in 1976. In 1994, Lee et al. characterized peptides released from the medaka-hardened egg envelope by the partial proteolytic action of medaka hatching enzyme and identified peptides containing significant amounts of γ-Glu-ε-Lys. These two results strongly suggested that a transglutaminase participated in chorion hardening because γ-Glu-ε-Lys formation is caused by transglutaminase. This possibility was strongly supported by results using cadavarine derivatives, which are competitive inhibitors of isopeptide crosslink formation (Oppen-Berntsen et al, 1990). The localization of the transglutaminse was indicated to be not in the cortical alveoli (granules) but in the egg envelope itself (Ha., et al 1995). In 1997 and 1998, Ha et al. purified two different forms of transglutaminase from unfertilized rainbow trout egg envelopes and characterized them as egg envelope hardening enzymes. Transglutaminase has been characterized and cloned from the livers of sea bream and chum salmon. However, the egg envelope form of the enzyme is apparently structurally different from the liver form.

It is obvious that the mechanisms of chorion hardening are controlled by at least two different enzymes. During the cortical reaction alveolin is discharged from the cortical granules into the perivitelline space and binds to the egg envelope, hydrolyzes the major

components of egg envelope, ZI-1 and -2 (in case of medaka egg). The transglutaminase coexisting in the egg envelope (chorion) then polymerizes hydrolyzed ZI-1 and -2 with ZI-3 by peptide crosslinking.

Acknowledgments

Professor Jerry L. Hedrick, Department of Animal Science at University of California, Davis, provided invaluable support through the experiments. I would like to express my thanks to Dr. LeAnn L. Lindsay, Department of Animal Science at University of California, Davis, for her invaluable advice in preparing this mini review. Thanks to the staff of the Nimbus California State hatchery of Department Fish & Game, California, who assisted in the sampling for Chinook salmon eggs and milt.

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Correct Citation of this Report is:

Sakai, Y., J.P. McVey, D. Jang, E. McVey, M.Caesar (eds.) 2005. AQUACULTURE AND PATHOBIOLOGY OF CRUSTACEAN AND OTHER SPECIES. Proceedings of the Thirty-Second U.S.- Japan Meeting on Aquaculture. Davis, California, 17,18 & 20th November, 2003. UJNR Technical Report 32. Silver Spring, MD: NOAA Research.

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