A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

First report on the occurrence of *Urosporidium* sp., a haplosporidian hyperparasite in Manila clam, *Ruditapes phillippinarum* in Korean waters

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GRADUATE SCHOOL

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First report on the occurrence of Urosporidium sp., a haplosporidian hyperparasite in Manila clam, *Ruditapes phillippinarum* in Korean waters

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국문요약

Haplosporidia 문의 단세포성 기생충 그룹에 속하는 *Urosporidium*은 해양 이매패류의 다양한 대형기생생물(macroparasite)의 내생기생충(endomicroparasite)으로 알려져 있다. 이 연구에서는 우리나라 조간대 바지락 (*Ruditapes philippinarum*)의 trematode 감염에 관한 조직병리학적 조사를 실시하여 2010년 봄 조사에서 처음으로 *Urosprodium*의 중복기생(hyperparasitism)을 확인하였다.

인천만, 태안, 남해안과 서해안의 28개 지역으로부터 총 1,074개의 바지락을 채집하였다. 전 채집지역에서 높은 digenetic trematode의 감염이 관찰되었으며, 감염률은 5%에서 70%까지 다양했으며, 평균적으로 32.5%의 감염률을 보였다. 조직학적 관찰시 감염된 바지락의 이생류 (digenea)는 3가지의 유생 발생 단계가 여러 기관에서 관찰되었다. 그 특징들은 외투막에서 피낭에 둘러싸여 있지 않은 (non-encysted) metacercaria, foot에서 피낭 유충 (encysted metacercaria), 생식소에서 sporocyst가 관찰되었다. 또한, 다양한 유생기의 trematode에 감염된 바지락의 감염 부위에서는 전형적인 혈구 침입과 염증반응이 관찰되었다. 그러나, sporocyst 단계에 감염된 숙주의 전형적인 감염증상은 생식소에서 발생하는 번식불능 (castration) 현상이었다. 감염된 바지락내 외투막 조직의 기생충성 침식 (parasitic erosion)과 이상증식(hyperplasia)은 다량의 metacercaria와 관련되어 있었다. 상기의 결과들로 digenetic trematode 감염은 바지락의 번식 능력과 성장을 저해할 것으로 사려된다.

Urosporidium sp.의 감염은 서해안과 남해안의 10개 지역 바지락 서식지에서 채집된 27마리의 바지락 체내 trematode의 기생으로 관찰되었다. 바지락 개체내 감염된 *Urosporidium* sp.의 감염율은 2.5%에서 24%의 분포양상을 나타내었다. 피낭에 둘러싸여 있지 않은 metacercaria와 cercaria형의 sporocyst에서 다양한 발생 단계의 *Urosporidium* sp.를 갖고 있었다. 변형체(plasmodium)와 접합포자(sporont) 시기의 *Urosporodium* sp.는 감염정도가 낮은 유생 trematode에서 중간정도의 trematode 감염을 나타내는

바지락에서 관찰되는 반면, 다량의 난형에서 구형 포자 (직경 3.1-6.3μm)를 갖는 sporocyst는 점진적으로 체형이 파괴되는 바지락에서 관찰되었다. *Urosporidium*의 포자가 성숙될수록 Ziehl-Neelsen's 염색시 항산성 성질 (acid-fastness)을 보였다. 이는 *Urosporidium*이 포자 형성을 완수하기 위해 trematode 체내를 이용하는 중복기생(hyperparatism)에 의해 trematode의 사멸을 유도하지만, 바지락에 대한 영향은 관찰되지 않았다.

I. ABSTRACT

Urosporidium is a group of unicellular parasites belonging to the phylum Haplosporidia, known as endomicroparasites of several macroparasites in marine bivalves. In this study, through a histopathological survey on trematode infection status of the adult commercial Manila clam, *Ruditapes philippinarum,* on tidal flats in Korea, the *Urosporidium* hyperparasitism was found for the first time in the spring of 2010.

A total of 1,074 clams were collected from 28 different locations in Incheon Bay and the Taean area off the south and west coasts of Korea. All the sites studied revealed a high infection of digenetic trematodes, with the average prevalence of 32.5% ranging from 5-70%. Histology also indicated three common larval stages of the digenea in the infected clams, which were observed in different organs as non-encysted metacercaria in the mantle, encysted metacercaria in the foot, and sporocysts in the gonad of the infected clams. Hemocyte infiltration and inflammation were typically observed at the vicinities of the parasitized loci within the host organs infected by various larval stages of trematodes. However, the most common pathologic symptom caused by infestation with sporocyst stages was castration of the host gonad. Parasitic erosion and hyperplasia of the mantle tissue were often elicited in association with the abundance of metacercaria in the infected clams. These observations suggested that digenetic trematode infestation may disturb reproductive capacity and growth of the clams.

The presence of *Urosporidium* sp. was recorded in the body cavity of trematodes parasitizing 27 clams, collected from ten clam beds on both the west and south coasts of Korea. The prevalence of these *Urosporidium* sp. infected clam populations varied from 2.5-24%. Unencysted metacercaria and cercaria-containing sporocysts harbored several different stages of *Urosporidium* sp. Plasmodial and sporont stages of *Urosporidium* sp. were observed only in slightly to moderately infected larval trematodes, while sporocysts containing a number of ovoid to round spores (3.1-6.3μm in diameter) were observed in the gradually devastated body of the hosts. As the spores become mature, they were shown to possess acid fastness when staining characteristically with Ziehl-Neelsen's stain. It was evident that *Urosporidium* sp utilized the

trematode body to complete their sporulation, and the hyperparasitism gave rise to the trematode's death but had no impact on the clams.

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I. INTRODUCTION

Manila clam farming has been becoming one of the most important commercial activities around the Korean peninsula, contributing about 33.2 million US dollars per year for the Korean economy for the past decades (FAO, 2011). A widespread species in the western Pacific Ocean, the Manila clam, *Ruditapes philippinarum*, has the ability to adapt to different types of tidal flats, and has been raised commercially along the western and southern coastlines of Korea (Chung *et al.* 2001; Park *et al.* 2005). Historically, after peaking at approximately 65,000 tons and stabilizing at over 45,000 tons, annual clam landings underwent a sharp decline, with only around 17,300 tons harvested remaining constant up to the present (FAO, 2011; Park *et al.* 2006). The sediment composition changes of clam habitats, over-fishing, and especially disease-associated mortalities were suspected as the most relevant causes (Park *et al.* 2001; Yang et al, 2010).

Amongst pathogenic biotic agents found in Manila clam, recent evidence has shown that bacteria and parasites are the two most common pathogens occurring in many aquaculture areas in Korea. *Vibrio tapetis*, for example, the marine bacterium responsible for the brown ring disease (BRD) (Paillard and Maes., 1994; 1995; Borrego *et al.*; 1996; Castro *et al.*, 1997) was first reported from clams inhabiting a western region of Korea (Park *et al.*, 2006). Up to now the BRD-caused impact on Korean clams has not been as evident as that on clams in European countries. However, more than one decade since the presence of *Perkinsus oselni*, a common pathogenic protistan parasite of marine bivalves, was found and then confirmed in Manila clams, this severe pathogen has exhibited a wide spatial distribution and considerable impact on many commercial and natural clam beds in Korea. A report of Park and Choi (2001), surveying *Perkinsus* infection status of clams, revealed that 18 out of 22 clam populations sampled from the west, south, and east coasts were infected by a high level (12 to 3,924,309) of *Perkinsus* cells/individuals). Infection by *Perkinsus* was known not only to give rise to growth reduction and reproductive retardation of the hosts but also to weaken the host immune system so that the affected animals become susceptible to other pathogens and found it difficult to cope with any unfavourable conditions. (Villalba *et al.*,

This thesis follows the style and format of *Journal of Fish Diseases*

2004; Park *et al.*, 2006; Choi and Park, 2010, Uddin *et al.*, 2010).

Along with the appearance and establishment of the *Perkinsus* protozoan parasite, *R. philippinarum*, like other bivalve species, has also been known to be a host providing "habitats" for parasitic larval stages in the life cycle of digenean trematodes before the adult individuals transferred to the bodies of vertebrates (Bowers *et al.*, 1996). Except for the egg and free-swimming miracidium larva existing in the environment, the later larval trematode stages comprising sporocysts, redia, cercaria and metacercaria usually need one or two intermediate molluscan hosts in which they can accomplish their metamorphosis and asexual reproductive processes (Schmidt and Roberts, 2000) (Figure 1). Several attempts have been conducted to investigate the species composition of the trematode fauna on originally parasitized clams, as well as to experimentally infected hosts, demonstrating that *Ruditapes philippinarum* can harbor more than seventeen species belonging to six discrete trematode genera (Cheng *et al.*, 1966; Kim & Chun, 1981; Bower *et al.*, 1992; Sohn *et al.*, 1996; de Montaudouin *et al.*, 2000; Lassalle *et al.*, 2007; Yanagida *et al.*, 2009; Han *et al*, 2009; Dang *et al*, 2009). Among them, three trematode species were reported to be parasitic in *R. philippinarum* in Korea, generating several pathological impacts such as follicles degeneration and deterioration of the infected connective tissues (Lee *et al.*, 2001; Ngo and Choi, 2004).

For many marine bivalves, however, infestation with digeneans is often associated with structural, behavioural, and physiological changes of the hosts. In cockles, according to Ching (1995), an abnormal shell-gaping phenomenon where the host valves became asymmetric, unable to fit closely to each other, was found when gymnophalid metacercariae resided in the cockles. Similarly, Bowsers *et al.* (1996) reported that incomplete closure of shell valves and inversion of *Cerastoderma* cockle species *in situ* in sand occurred because of the excessive decomposition of host ligament fragments and impaired periostracum under the stimuli of unencysted metacercariae. With respect to internal alterations of trematode-parasitized bivalves, parasitic castration (Laucker, 1984) has been known as the most common parasite-induced syndrome observed in mussels, oysters, and clams (Santos and Coimbra, 1995; Silva *et al.*, 2002; Valderrama *et al.*, 2004).

Concomitantly with these physical variations, several bivalves also presented a variation of hemocyte parameters, growth reduction and heavy mortality associated with high trematode burden (Santos and Coimbra, 1995; Silva et al., 2002). As a consequence, it was believed that impact of digenean parasitism on various bivalves appeared to be involved with different trematode species and their diverse developmental stages.

Interestingly, besides being pathogens to many marine bivalves, the digeneans have been known to be subjected to several sporozoans, particularly Haplosporidia protozoa group. Although many species in order Haplosporidia are often concerned as pathogenic unicell parasites, reported in association with serious mortalities in several eastern oyster species, there is only *Urosporidium* often being parasites of another parasites or called “hyperparasites” (Anderson *et al.*, 1993; Burreson and Ford, 2004; Carballal et al, 2005). The blue crab *Callinectes sapidus*, the mollusk *Rissoa splandida*, and the clam *Abra ovata* was documented as the secondary hosts of trematode larva infected by *Urosporidium* sp. (DeTurk, 1940; Zaika and Dolgikh, 1963; Perkins, 1971; Ormières et al, 1973).

During a field survey on the heath status of *R. philippinarum* populations commercially culturing along the west and south coast of Korea, we first found hyperparasitism of *Urosporidium* sp. invading into the trematodes that commonly parasitize the clams. In this study, through reporting on trematode infection status in the clams, morphological features, pathogenicity, and several histochemical components of *Urosporidium* sp. were also discussed.

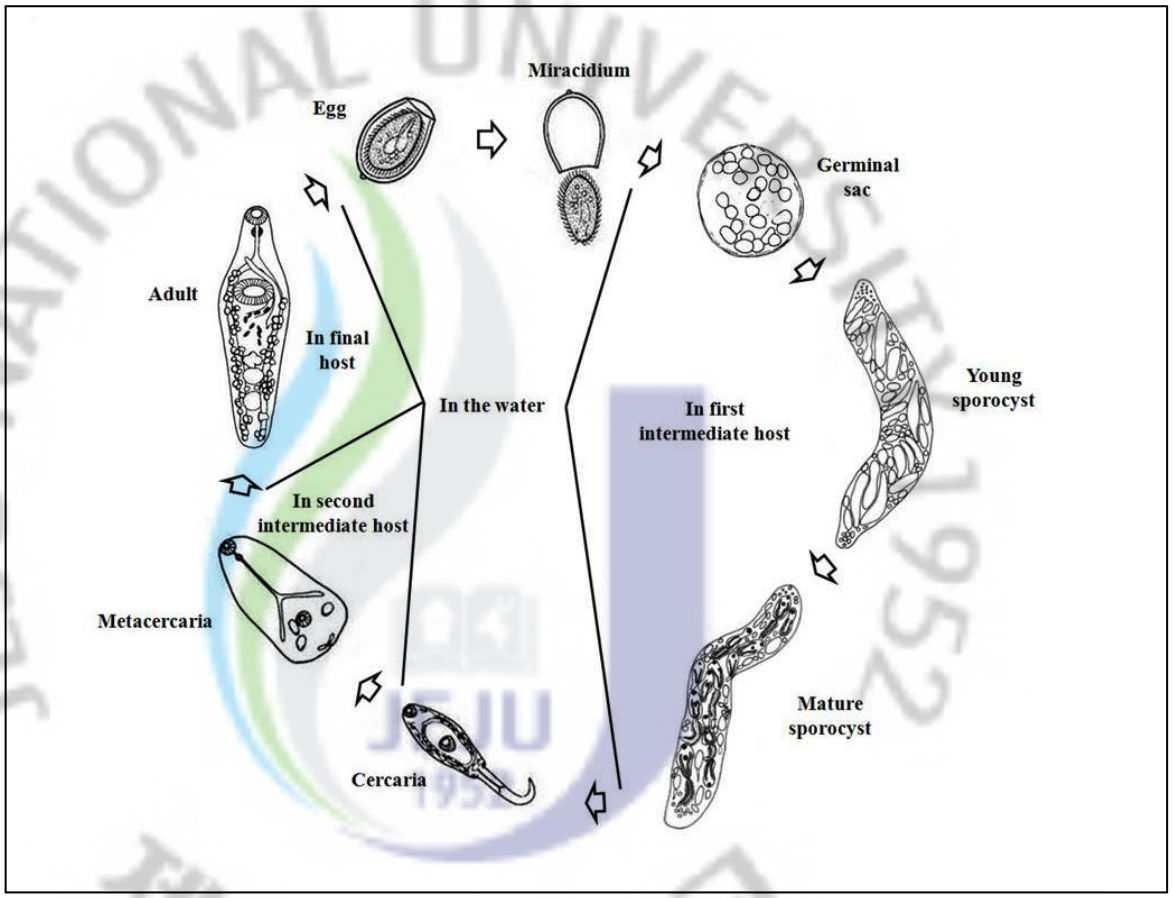

Fig.1. A typical life cycle of trematode parasite (After Möller and Anders, 1986; Abdallah et al, 2009; Rohde, 2011)

II. MATERIALS AND METHODS

2.1. Sample collection

During the month of April, 2010, adult clams 26.5–37.6 mm in shelllength were sampled at various locations (126°12´-127°22´E, 34°63´-37°25N) around the Korean peninsula. Of the 28 sampling sites surveyed, 27 sites are scattered along the west coast facing the Yellow Sea while one is in the southern coastal region on the South Sea (Korea Strait) (Fig 2). All the locations surveyed are commercial clam beds, composed of the three main kinds of sediment: muddy sand, sandy gravel, and sandy silt. For each site, 25-40 live clams were randomly sampled for evaluation of condition index and histopathological investigation. Data regarding study region, geographic coordinates, and sediment type relative to each sampling site is presented in Table 1.

2.2. Condition index

In the laboratory, after measurement of maximum shell length, the whole soft body of each specimen was removed and weighed before preparation for histological processing. Condition index (CI), or the overall physiological status of each clam, was calculated as described by Park *et al.*, 2006, i.e. by the proportion of wet tissue weight to dry shell weight.

2.3. Histopathology

A 2 mm-transverse section containing a part of the gill, mantle, gonad, digestive gland, and foot was excised in the middle of each clam body and fixed in Davidson's solution for 24 hours. The excised tissue was dehydrated in alcohol, embedded in paraffin, and sectioned at 6 μm thick. Non-paraffined sections were stained with Harris' Hematoxylin and Eosin (H&E), and then examined for parasites by regular light microscopy.

After the presence of hyperparasites was histologically identified, a few paraffin-embedded segments which had been cut once were selected for histochemical study.

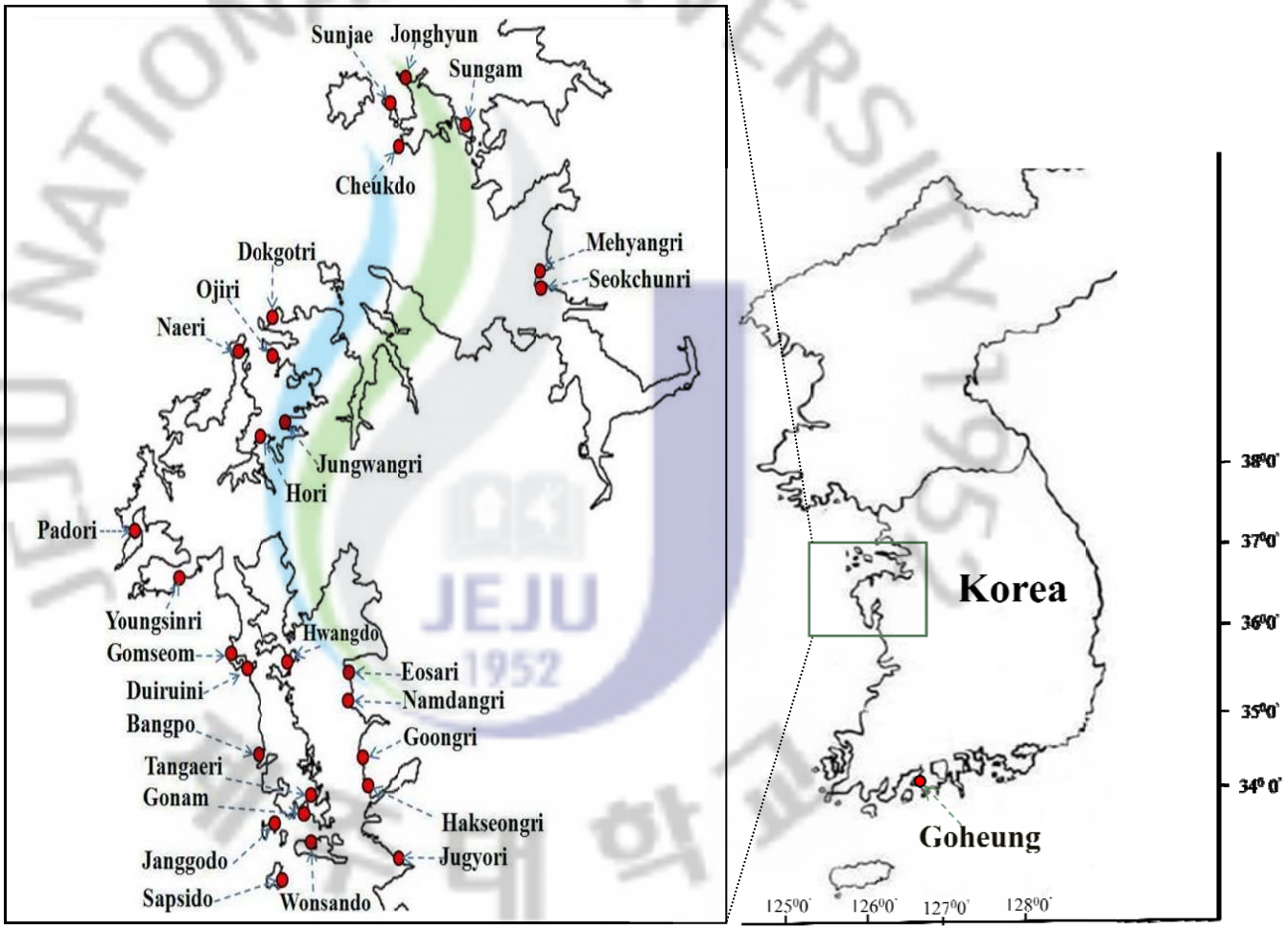

Fig.2. Map showing the clam sampling sites in Korea Waters

Table 1. . Bottom types of the intertidal clam sampling locations

Site	Sampling date	Region	Location	Sediment type
Sunjae	02-Apr	West coast	E126°52′, N37°25′	Muddy sand
Cheukdo	02-Apr	West coast	E126°51′, N37°23′	Muddy sand
Jonghyun	26-Apr	West coast	E156°57′, N37°26′	Muddy sand
Sungam	02-Apr	West coast	E126°62′, N37°22′	Muddy sand
Mehyangri	03-Apr	West coast	E126°75′, N37°04′	Muddy sand
Seokchunri	03-Apr	West coast	E126°74′, N37°02′	Gravelly sand
Ojiri	13-Apr	West coast	E126°35′, N36°95′	Gravelly sand
Dokgotri	13-Apr	West coast	E126°36′, N37°00′	Gravelly sand
Hori	28-Apr	West coast	E126°34′, N36°85′	Gravelly sand
Naeri	12-Apr	West coast	E126°29′, N37°16′	Gravelly sand
Padori	12-Apr	West coast	E126°12′, N36°42′	Gravelly sand
Hwangdo	11-Apr	West coast	E126°22′, N36°36′	Muddy sand
Gonam	10-Apr	West coast	E126°41′, N36°41′	No data
Tangaeri	10-Apr	West coast	E126°40′, N36°30′	Sandy silt
Bangpo	10-Apr	West coast	E126°33′, N36°50′	Gravelly sand
Gomseom	11-Apr	West coast	E126°29′, N36°60′	Gravelly sand
Duiruini	11-Apr	West coast	E126°32′, N36°59′	Sandy gravel
Youngsinri	12-Apr	West coast	E126°23′, N36°70′	Muddy sand
Hakseongri	14-Apr	West coast	E126°49′, N36°45′	Muddy sand
Sapsido	15-Apr	West coast	E126°37′, N36°33′	Muddy sand
Janggodo	15-Apr	West coast	E126°34′, N36°40′	Muddy sand
Wonsando	15-Apr	West coast	E126°41′, N36°37′	Muddy sand
Jugyori	02-Apr	West coast	E126°52′, N36°37′	No data
Namdangri	27-Apr	West coast	E126°47′, N36°54′	Muddy sand
Goongri	14-Apr	West coast	E126°48′, N36°61′	Gravelly sand
Eosari	14-Apr	West coast	E126°47′, N36°56′	Muddy sand
Goheung	25-Apr	South coast	E127°22′, N34°63′	No data
Jungwangri	02-Apr	West coast	E126°37′, N36°87′	No data

2.4. Histochemical preparation

Each hyperparasitized specimen selected was carefully re-sectioned from the previously sectioned paraffin segments to get a series of 5-6 μm thick slices. The re-sectioned sample was completed deparaffinized in xylen and water hydration through descending concentrations of ethyl alcohol (ETOH) before being stained by one of the following methods. Ziehl's carbol fuchsin (Farley, 1965) was employed to detect acid-fastness and the periodic acid-Shiff test (PAS test) for carbohydrate detection (Howard *et al*, 2004).

2.5. Prevalence, intensity of infection, and size measurements of parasites

The presence of each particular parasite species in the host species, namely trematodes in clams and hyperparasites in trematodes, at every surveyed site, was assessed by using histology and expressed through infection prevalence that was determined as the percentage of infected hosts (Bush *et al.*, 1997). Trematode prevalence stands for the percentage of clam individuals infected by each of any parasite types.

Regarding infection of clams by trematode larvae, four stages of trematodes were separated, including (1) non-encysted and (2) encysted metacercaria, (3) germinal ball-sacs (sacs that contains only germinal balls), and (4) cercaria sporocysts (sacs or sporocysts that comprise cercariae and germinal balls). Infection prevalence relative to each trematode larval stage was determined as the definition of the total trematode infection, i.e. the proportion of clam individuals infected by one relevant type of trematode larvae to the number of clams examined. For the abundance of metacercaria in each infected clam, the number of metacercariae counted on each entire histological slide was considered as infection intensity.

For size measurements of parasites, 'Image J' software (http://rsb.info.nih.gov/ij/) was used to measure the size of all parasites found and photographed at different magnifications.

III. RESULTS

3.1. Biometric data and condition index

Biometric data and condition index of samples from 28 study sites, calculated from a total of 1074 clams, were summarized in Table 2. The shell length of clam individuals varied, with a mean ±STDEV value of 33.38±4.09 mm, ranging from 19.20 to 51.30 mm. The tissue wet weight of the clams showed a mean ±STDEV weight of 2.2171±1.1100g, differing from 0.4004 to 8.3771g among the sites. Therefore, the clams showed a wide range of body size, with a mean ±STDEV value of 0.6068±0.1655, varying from 0.1563 to 1.2142.

Among the clams examined, the individual group with 32-34mm in shell length was most common, about 43% of all clams analyzed. In term of CI, the most common were the clam classes with CI of 0.5-0.6 (57.1 %), followed by groups with CI<4(21.4%) and CI > 6 (21.4%).

3.2. Trematode infestation

In this study, the clams presented a relatively high frequency of trematode infection. From the clams examined, 349 individuals, a prevalence of 32.5 % were parasitized. Of the infected clams, the metacercarial stage of the trematodes was the most abundant, infecting 100% of the parasitized clam specimens, followed by the sporocyst stage, a prevalence of 21.9%, including germinal-ball sacs (13.8%) and cercaria sporocysts (8.1%).

At the population level, histological results, especially, also showed that no clam bed was free from trematode infection. The total infection prevalence among the sampling sites differeed greatly, ranging from a minimum level of 5% observed in clams from Jangodo to a maximum of 70% in the Hwangdo clams (Table 3). Furthermore, the total prevalence of parasitized clam populations from these sites seemed to have no correlation with their location.

3.2.1 Digenetic metacercarial infestation

Through squash examination and histological observation on several live clam specimens

from the sampled sites, two main different types of metacercariae were seen on different parts of the body of the infected clams. Live encysted metacercariae (diameter ± standard deviation, 316.6±21.0μm, N=10) were round, whitish, and found mostly in the clam foot. The fluke body was enclosed in a thin cyst wall, embedded by a visible filamentous layer of the host tissues. At the end of the metacerarial cysts, sometimes, several morphological details such as suckers, numerous collar spines, and even excretory granules were able to be observed under a light microscope (Fig 3A). On the contrary, the second type of metacercaria or unencysted larva exhibited a more apparent morphological structure: the body was oval shaped, with a conspicuous, sub-terminal large sucker and a ventral small sucker. Granular excretory concretions were distributed at both lateral sides, expanding from a position under the oral sucker to the posterior end of the fluke body. These infective larvae were active and mainly encountered in the mantle cavity of their clam hosts (Fig 4A). Histologically, metacercarial bodies were spherical to round-shaped forms covered by an eosinophilic wall that had a consistent thickness. The outer wall layer was regularly serrated, attached by an internal cellular basement membrane (Fig 4B & 4C).

In histological cross-sections, response of the infected clams to two types of digenean larvae was dissimilar among the infected host individuals and the parasitized organs. In the mantle, the digenean larvae were often found to be parasitic on the surface of the mantle epithelium, particularly in an abnormally enlarged portion of this organ (hyperplasia). The infected location was often eroded in association with the presence of the parasite (Fig 4), causing a conspicuous hollow separating the parasite and host tissue. Deformation of the host tissue was also observed at the loci where the larval oral sucker was feeding on host tissues. Hemocyte infiltration and the formation of an opaque, mucus-like substance covering the parasite were often detected in correlation with reduction of mantle epithelial thickness. Nevertheless, the muscular tissues of the foot, gonad, and digestive gland appeared to be more damaged than those of the mantle, as they were parasitized by encysted metacercariae. The most common histological observation was the replacement of a large part of the host tissues by parasitic cysts, mostly at the margins of the host foot. Encircling each cyst was a non-cellular thick wall that separated the cyst body and the surrounding host tissue by an empty space. Often, marked hemocytic inflammation, which may or

may not be melanized, was often present in the host tissues around the parasitic cysts (Fig 3.1 B&C). Such effects were also seen on the host connective tissue adjacent to the digestive tubes where metacercarial encystment was occurring. In some specimens, an inflammatory response of the clam was observed on follicular tissues of the nearly empty gonads when parasites were present (Fig 3.2). However, in a few cases of infected hosts, digestive diverticular atrophy, muscular necrosis, and no lesions were also recorded together on the same clam individual embedded with metacercarial cysts.

The results of microscope slide examination demonstrated that in this study metacercarial infection, accounting for two parasitized organs of clams, was fairly high. Of 1074 clams surveyed, 299 individuals were affected by metacercaria (27.9 % prevalence) with an average abundance of 2.9 metacercariae per clam (ranging from 1-25 parasites per clam. The prevalence of parasitized clams per sampling site differed between 5-67%, in which concomitant infestation, involving both the organs, was predominant at many sampling sites rather than a single infection.

In terms of the difference in digenean metacercarial infection between the mantle and foot of the host, the overall prevalence of clams infected by metacercariae in the mantle was 22.5 % (N=242), about threefold higher than those where the parasites were located in the foot (0.07%, N=75). Mean abundance of metacercariae in the mantle and foot was 3.1 and 1.65 parasites per clam, respectively. The variation in occurrence and abundance of metacercariae in respect to each larval stages across the sampling sites is exhibited in Table 4.

Table 2. Biometric data of the clam populations analyzed. N: number of clams analyzed; SL: mean of shell length in mm ± SD; TWWT: mean of tissue wet weight in gram ± SD; CI: mean of condition index± SD; SD: standard deviation.

Site	N	SL	TWWT	CI
Sunjae	40	33.34 ± 1.96	1.819 ± 0.480	0.519 ± 0.114
Cheukdo	40	32.48 ± 2.70	1.641 ± 0.368	0.494 ± 0.086
Jonghyun	40	36.58 ± 1.87	2.175 ± 0.537	0.481 ± 0.101
Sungam	40	33.65 ± 1.85	1.729 ± 0.404	0.418 ± 0.100
Mehyangri	40	33.57 ± 2.84	1.900 ± 0.653	0.519 ± 0.095
Seokchunri	40	29.64 ± 4.79	1.483 ± 0.685	0.593 ± 0.090
Ojiri	40	33.24 ± 2.01	1.853 ± 0.334	0.612 ± 0.134
Dokgotri	40	33.13 ± 1.88	1.620 ± 0.340	0.426 ± 0.062
Hori	40	37.66 ± 2.35	3.625 ± 0.960	0.719 ± 0.122
Naeri	40	32.47 ± 1.30	1.399 ± 0.136	0.479 ± 0.092
Padori	40	31.59 ± 1.97	1.328 ± 0.253	0.430 ± 0.054
Hwangdo	40	35.45 ± 3.10	3.206 ± 0.746	0.775 ± 0.079
Gonam	40	35.20 ± 2.59	2.176 ± 0.584	0.550 ± 0.093
Tangaeri	40	36.13 ± 2.30	2.500 ± 0.605	0.540 ± 0.089
Bangpo	30	31.73 ± 3.23	2.015 ± 0.587	0.669 ± 0.092
Gomseom	29	33.02 ± 2.39	2.257 ± 0.796	0.651 ± 0.122
Duiruini	30	36.44 ± 3.16	4.124 ± 1.196	0.834 ± 0.128
Youngsinri	40	36.94 ± 2.10	3.190 ± 0.656	0.630 ± 0.071
Hakseongri	40	37.88 ± 2.54	3.176 ± 0.677	0.602 ± 0.121
Sapsido	40	40.58 ± 3.15	3.985 ± 0.940	0.588 ± 0.106
Janggodo	40	39.26 ± 3.68	3.994 ± 0.136	0.630 ± 0.113
Wonsando	40	33.16 ± 1.95	2.087 ± 0.469	0.655 ± 0.089
Jugyori	40	32.36 ± 1.58	1.989 ± 0.325	0.648 ± 0.068
Namdangri	25	39.26 ± 3.55	4.721 ± 1.384	0.788 ± 0.109
Goongri	40	38.97 ± 1.86	4.859 ± 0.759	0.971 ± 0.103
Eosari	40	33.15 ± 1.20	1.991 ± 0.248	0.792 ± 0.130
Goheung	40	37.81 ± 2.22	3.524 ± 0.803	0.663 ± 0.130
Jungwangri	40	33.15 ± 1.20	1.991 ± 0.248	0.632 ± 0.069

Table 3. Mean prevalence and intensity of trematode infection at the survey sites. Trematode prevalence was counted as a percentage of clams infected by each of any parasite types. Infection prevalence of clams corresponding with each trematode larval stage was determined as a proportion of clam individuals infected by one relevant type of trematode larvae to the number of clams examined. Metacercaria infection intensity was determined as the mean number of metacercariae found in whole histological slides. Min-Max: minimum and maximum quantity of metacercariae

Site	N	Trematode Prevalence	Metacercaria		Sporocyst Prevalence
			Prevalence	Intensity (Min-Max)	
Sunjae	40	37.5	30.0	2.2 (1-4)	10.0
Cheukdo	40	40.0	30.0	5.5 (1-32)	15.0
Jonghyun	40	32.5	27.5	1.6 (1-3)	5.0
Sungam	40	25.0	20.0	1.9 (1-3)	5.0
Mehyangri	40	35.0	32.5	1.8 (1-4)	5.0
Seokchunri	40	40.0	37.5	2.5 (1-5)	7.5
Ojiri	40	30.0	25.0	5.4 (1-16)	0.0
Dokgotri	40	20.0	20.0	1.7 (1-2)	0.0
Hori	40	25.0	15.0	1.3 (1-2)	10.0
Naeri	40	25.0	22.5	1.9 (1-3)	2.5
Padori	40	7.5	5.0	1.0	2.5
Hwangdo	40	70.0	67.5	4.0 (1-22)	12.5
Gonam	40	47.5	47.5	3.7 (1-10)	7.5
Tangaeri	40	52.5	40.0	1.4 (1-3)	10.0
Bangpo	30	16.7	13.3	2.0 (1-5)	3.3
Gomseom	29	37.9	34.5	1.1 (1-2)	6.9
Duiruini	30	43.3	43.3	10.1 (1-25)	2.5
Youngsinri	40	60.0	62.5	1.5 (1-3)	7.5
Hakseongri	40	22.5	20.0	1.5 (1-3)	2.5
Sapsido	40	22.5	15.0	2.0 (1-4)	10.0
Janggodo	40	5.0	2.5	1.0	2.5
Wonsando	40	32.5	22.5	1.1 (1-2)	7.5
Jugyori	40	25.0	22.5	2.4 (1-11)	2.5
Namdangri	25	48.0	36.0	6.8 (1-21)	0.0
Goongri	40	12.5	10.0	2.5 (1-4)	2.5
Eosari	40	32.5	27.5	1.6 (1-2)	5.0
Goheung	40	35.0	25.0	2.3 (1-4)	15.0
Jungwangri	40	35.0	30.0	1.6 (1-4)	5.0

Table 4. Prevalence and infection intensity of the larval stages of trematodes in the different host organs. The value in parenthesis is the minimum and maximum quantity of metacercariae. GBS: Sac contains only germ balls; CS: sporocyst contains cercariae.

Site	N	Mantle metacercaria		Foot metacercaria		GBS	CS
		Prevalence	Intensity	Prevalence	Intensity	Prevalence	Prevalence
Sunjae	40	30.0	2.2 (1-4)	0.0	0.0	10.0	0.0
Cheukdo	40	27.5	5.9 (1-32)	5.0	1.0	0.0	15.0
Jonghyun	40	22.5	1.3 (1-3)	10.0	1.5 (1-2)	0.0	5.0
Sungam	40	17.5	2 (1-3)	2.5	1.0	0.0	5.0
Mehyangri	40	27.5	1.9 (1-4)	7.5	1.0	2.5	2.5
Seokchunri	40	32.5	2.8 (1-5)	5.0	1.0	7.5	0.0
Ojiri	40	25.0	5.4 (1-16)	0.0	0.0	0.0	0.0
Dokgotri	40	20.0	1.7 (1-2)	0.0	0.0	0.0	0.0
Hori	40	15.0	3.5 (1-12)	0.0	0.0	10.0	0.0
Naeri	40	22.5	1.9 (1-3)	0.0	0.0	2.5	0.0
Padori	40	5.0	1.0	0.0	0.0	0.0	2.5
Hwangdo	40	37.5	4 (1-18)	52.5	1.8 (1-4)	10.0	2.5
Gonam	40	35.0	3.7 (1-10	17.5	1.7 (1-4)	2.5	5.0
Tangaeri	40	37.5	1.3 (1-3)	2.5	2.0	5.0	5.0
Bangpo	30	10.0	2.3 (1-5)	3.3	2.0	0.0	3.3
Gomseom	29	31.0	1.1 (1-2)	3.4	1.0	3.4	3.4
Duiruini	30	43.3	10.1 (1-25)	0.0	0.0	2.5	0.0
Youngsinri	40	10.0	1.2 (1-2)	52.5	1.6 (1-3)	5.0	0.0
Hakseongri	40	17.5	1.6 (1-3)	2.5	1.0	2.5	0.0
Sapsido	40	10.0	1.7 (1-4)	7.5	1.7 (1-2)	7.5	2.5
Janggodo	40	2.5	1.0	0.0	0.0	0.0	2.5
Wonsando	40	17.5	1.0	7.5	1.0	7.5	0.0
Jugyori	40	17.5	2.9 (1-11)	5.0	1.0	0.0	2.5
Namdangri	25	36.0	6.8 (1-21)	0.0	0.0	0.0	0.0
Goongri	40	7.5	1.7 (1-2)	5.0	2.5 (1-4)	2.5	0.0
Eosari	40	27.5	1.6 (1-2)	0.0	0.0	5.0	0.0
Goheung	40	25.0	2 (1-4)	25.0	2 (1-4)	12.5	2.5
Jungwangri	40	30.0	1.6 (1-4)	0.0	0.0	5.0	0.0

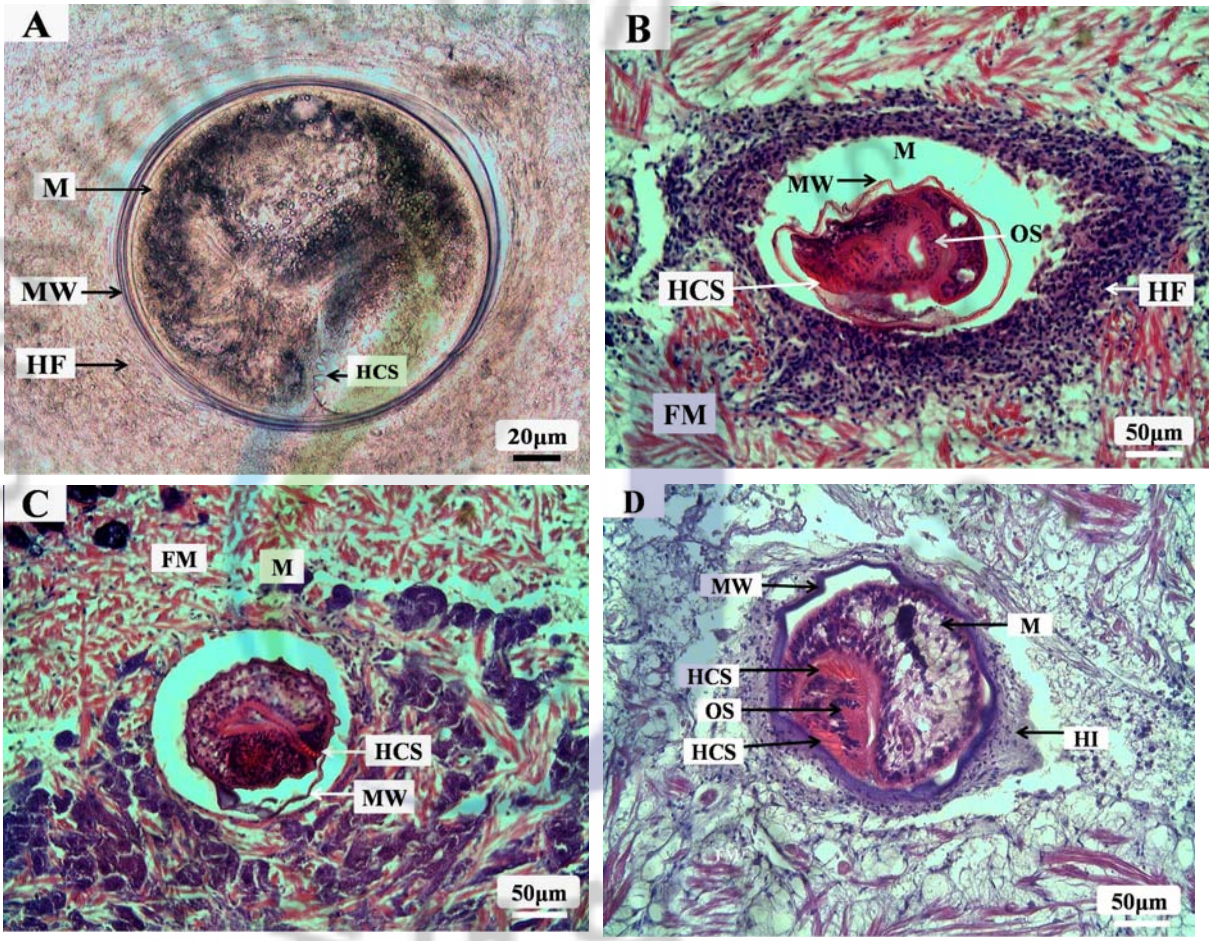

Fig.3.1. Metacercariae in foot muscle tissue of *R. philippinarum*. A: Live metacercaria; B, C, D: Metacercariae in histological sections. M: Metacercaria, MW: metacercaria wall, HF: host foot; HCS: head collar spine; HF: hemocytic infiltration.

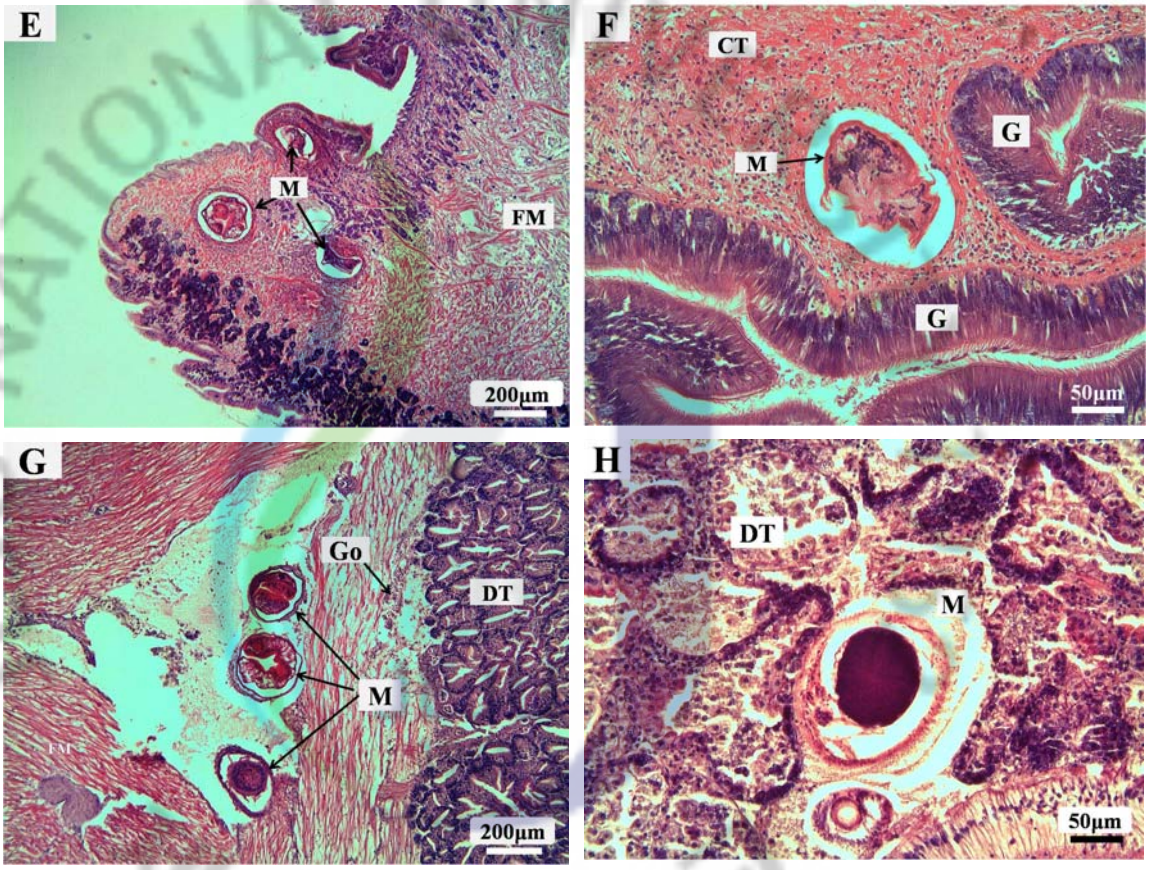

Fig.3.2. Metacercariae in the foot and other organs of *R. philippinarum*. E: Encysted metacercariae in the foot; F: Metacercaria in connective tissue of the gut; G: Encysted metacercariae in gonad; H: Metacercaria in digestive gland; M: Metacercaria; FM: foot muscle; Go: Gonad; DT: Digestive tube.

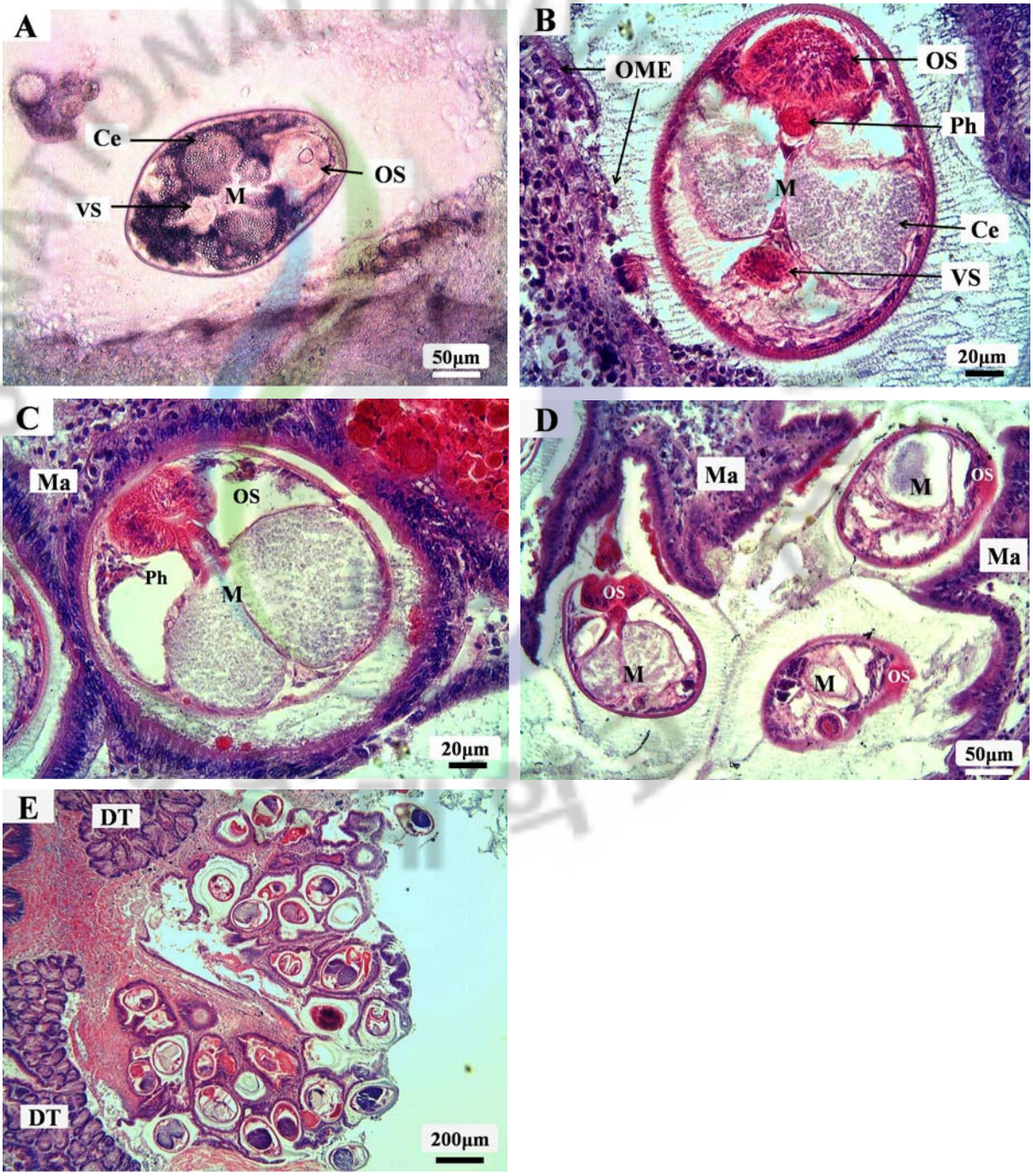

Fig.4. Unencysted metacercariae in the mantle cavity of *R. philippinarum*. A: Live metacercaria; B, C, D, E: Metacercariae in histological sections. M: Metacercaria, Ce: Caceum; OS: Oral sucker; VS: Ventral sucker; OME: outer mantle epithelium; Ph: Pharynx

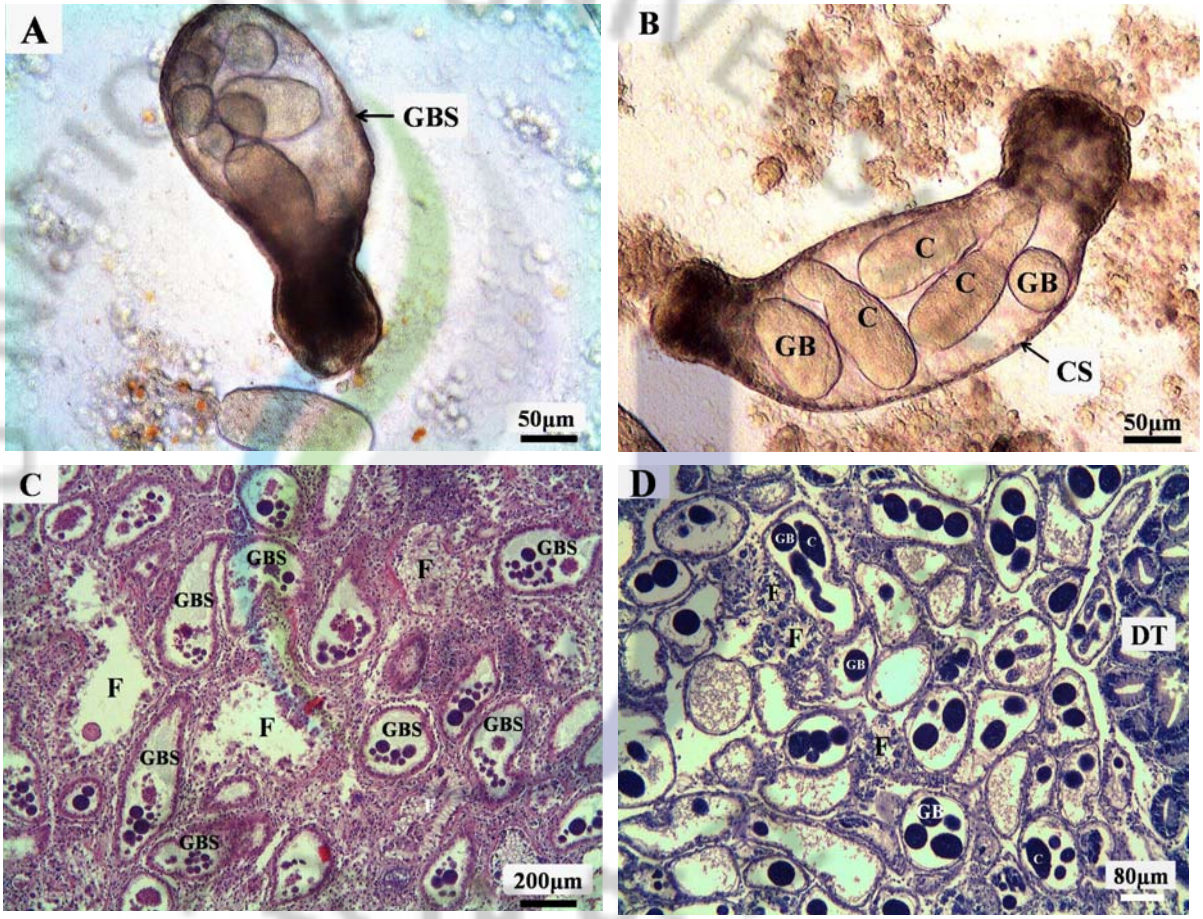

Fig.5.1. Trematode sporocysts in the gonad tissue of *R. philippinarum*. A: Live sporocyst sac containing several germinal balls (GB); B: Live early mature sporocyst including germinal balls (GB) and developing cercariae (C); C: Cross-section of the follicule (F) invaded by germinal ball sacs (GBS); D: Early mature sporocyst occupying the follicule.

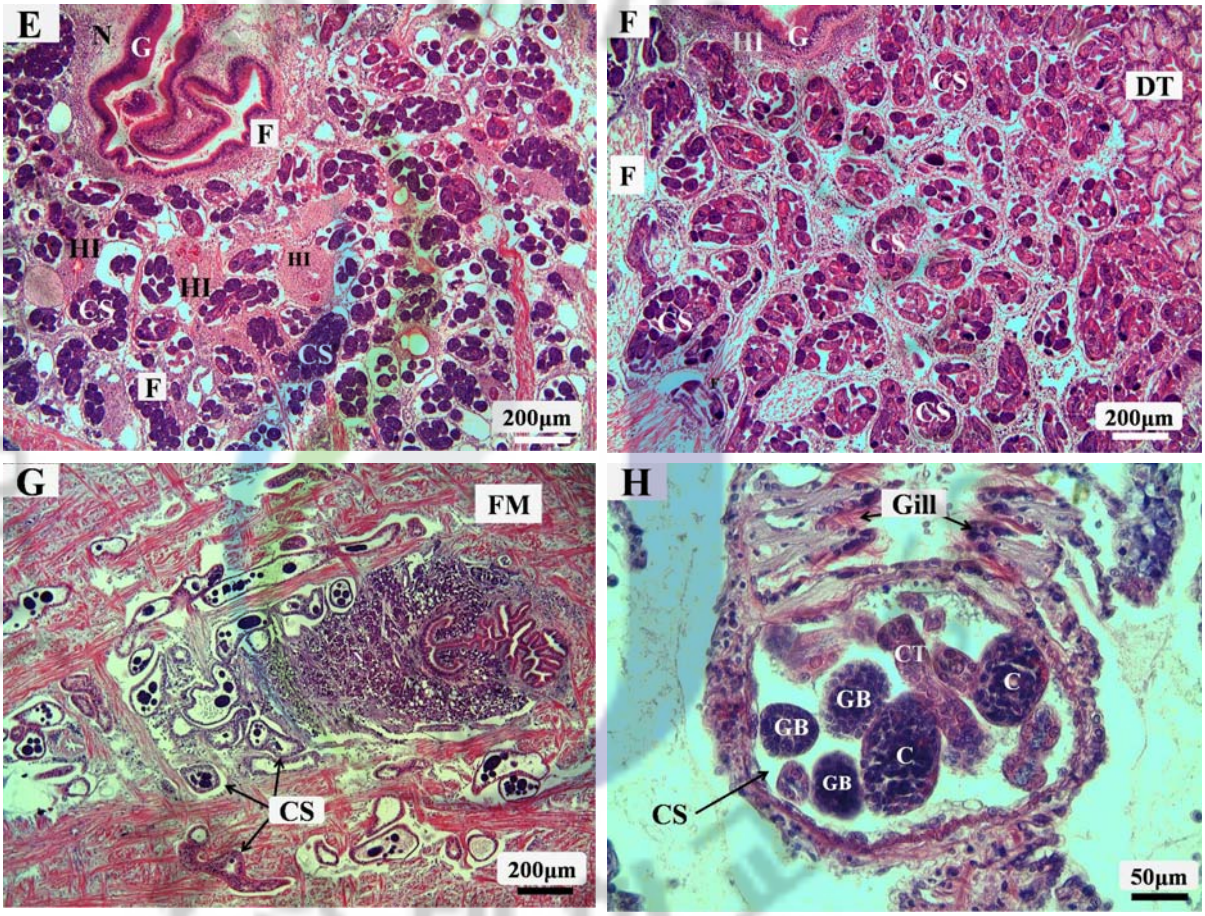

Fig.5.2. Histological sections showing digenetic sporocyst infection in different types of tissue clams. E: Early mature sporocysts in gonad and connective tissue surrounding the gut of *R. philippinarum*; F: Fully mature sporocysts colonizing the entire gonad; G: Sporocysts parasitizing the foot muscle; H: trematode sporocysts in the gills

(C: Cercaria; CS: Cercaria sporocyst; DT: digestive tube; GB: germinal ball; HI: hemocytic infiltrate; G: gut.

3.2.2 Digenetic sporocyst infestation

There were various types of trematode sporocysts detected through fresh tissue squash preparation and histological observation. Furthermore, two main kinds of parasite larvae were grouped based on their morphology and affinity maturation as observed when examined. Both stages showed a transparent, sausage-like body enclosed by a thin wall and harbouring different stages of digenetic trematode. The germinal-ball sac (Fig 5.1A), presumably was in the initial developmental stage of trematodes after the free-swimming miracidium larva penetrated the host. A striking characteristic of this stage, causing it to differentiate from the second sporocyst type, was that it is composed only of the early embryos (germinal balls), which may metamorphose into either rediae, cercaria, or daughter sporocysts. In histological cross-sections, the germinal balls were identically basophilic, spherical to round, and had no appendages such as tail and eyespots (Fig 5.1A).

The morphology of the second sporocyst type was completely different from that of the first one. In fresh specimens, mature sporocysts were larger and characterized by an opaque thinner wall in which developing cercariae were densely packed together in the anterior half of the sporocyst body (Fig 5.1B). The maturity degree of these sporocysts varied according to the number, size, and maturity of cercariae inside their body. However, in this study the sporocystes whose cercariae showed a body with the emergence of a tail were considered as the second sporocyst type.

Histological sections of these sporocysts provided some more apparent evidence of morphological differentiation in comparison with the first, which is particularly highlighted in the difference in density and size of the components contained inside their bodies. Namely, the germinal-ball sacs (first produced sporocyst type) are small, thick-walled, and composed of homogeneously basophilic stained oval forms (germ balls). The cercaria sporocysts (post-types of germinal-ball sacs) were often found as the large tubules including mostly dense cercariae at different stages of development and few germinal balls. A typical cercaria was oblong or elongated-oval with an enlargement of the anterior head and a vertical tail at the posterior end. In histological slides, its different parts were heterogeneously stained with H&E (Fig 5.2).

Microscope observations on infected clams revealed that the most severe infection by digenetic sporocyst larvae often took place in the gonad. At low infection level, in which several germinal sacs penetrated the gonadal tissues and grew near the follicule, normal gametogenesis was in process. No clear sign of parasite impact as well as the host response could be seen. However, along with the increase of the sporocyst burden was a decrease or disappearance of the gonadal areas where there used to be a network of acini (Fig 5.2). The gonad became barren due to displacement and compression caused by sporocyst proliferation. Interestingly, the level of barrenness in the infected gonad appeared to have a relationship with the two stages of sporocysts. The number and width of follicles in the clams whose gonads were invaded by germinal sacs were often larger than that of those whose gonads were colonized by mature sporocysts. Also, while interfollicular spaces of the gonadal tissues still exist and are connected at each other in case of germinal sac infection, fluke sporocyst infection was very little or rarely recorded. At very heavy infection, in which parasite larvae attacked the entire area of the gonad and spread over other organs, such as the digestive gland, mantle, and gill, no reproductive tissue was seen. At such infected organs, mature sporocysts were usually found rather than germinal sacs (Fig 5.2 G&H).

Although overall prevalence of trematode sporocyst larva in the specimens in this study was quite low, 6.05 % (N=65), there were only three sampling sites uninfected by this stage of trematode development (Table 3). Prevalence at the other sites varied between 2.5 to 15 %, with maximum prevalence observed at 2 sites while the minimum prevalence was recorded at 7 sites.

3.3. Hyperparasite infestation

Several different stages in the life cycle of Haplosporidian endoparasites, presumably *Urosporidium*, was found in trematode parasites, infecting 27 individual clams (prevalence 0.02%) collected from 10 clam beds in the surveyed region in this study. Except in Sapsido, where trematode sporocysts and metacercaria were found to be infected with *Urosporidium* microparasites, the others only recorded hyperpasitism in metacercariae. Non-encysted metacercariae in the mantle was one of two metacercarial stages in which *Urosporidium* was seen;

several were observed encapsulated by the mantle connective tissues while the rest were free on the matrices of the mantle.

The presence of the microparasites within digenean metacercariae was readily seen in histological sections. The residual shape of the digenean hosts, at low infection rates by the hyperparasite *Urosporidium* plasmodia, was violet, solid, multinucleate-containing spheres associated with the space between the oral sucker and ceca, either detached or attached to the inner basement membranes (Fig 6A). The size and number of nuclei within each plasmodial cyst in each infected metacercaria were varied. For transparent and refringent plasmodia, 3 to 48 nuclei within a plasmodium were common. However, for the other plasmodia in which the internal morphology was undistinguishable due to the dense growth and overstaining of their detail, the number of nuclei was impossible to examine. Relative to quantitative multiplication of plasmodia was the prominent disorganization of the metacercaria (Fig 6B).

In contrast to the plasmodial shape, the sporocyst was the next stage of *Urosporidium* development seen, at which immature, ovoid spores were produced and still packed tightly together within a thick-walled cyst. Each of these relatively translucent spores was structured by a distinct golden envelope enclosing an eosinophilic sporoplasm at the centre (Fig 6C). Increase in the color intensity of the spore envelope and particularly the change of endosporoplasm could be the morphological signals of spore maturity levels.

Though still enclosed within the sporocyst wall, mature spores appeared to have the characteristic morphology of the Haplosporidia. Basically, the morphological characteristics of a mature spore was similar to that of the immature one that was described above, such as being comprisied of endosporoplasm and a yellow envelope. Nevertheless, in case of a typical mature spore, the endosporoplasm within each spore became apparently darker and eccentric because one of its ends is closely attached to cyst wall. Thus, a large clear cytoplasm could be seen at the opposite side of the spore body (Fig 6D). The spore envelope also became much more yellow, resulting in the sporocyst containing such spores with a strikingly sweetsop-like shape (Fig 6B&C). These sporocysts were flexible in size and commonly included more than a hundred internal spores for each. However the number of sporocysts within infected metacercaria and the number of spores

existing within each sporocyst changed between metacercaria and sporocysts.

In many cases, sporocysts also appeared to have many spores that had no endosporoplasm. These yellow, empty spores were variable in shape, but the most popularly observed morphology was cubic, occurring inside metacercaria individuals with intensified hyperparasite infection. However, it was a fact that although the empty spore-containing clusters were interspersed inside the metacercariae, the general morphology of infected metacercariae was kept constant until the spore encystment was observed.

Free mature spores was the final developmental stage of *Urosporidium* recorded. The spores with average size of 3.32-6.18μm in length was no longer tightly clustered together within a sporocyst wall. The size of infested metacercariae seemed to be larger than that of uninfected metacercariae and infected metacercariae harboring *Urosporidium* sporocysts. Furthermore, the body structure of the infected metacercariae appeared to be damaged because their body cavity was completely colonized by massive numbers of both empty and solid spores (Fig 6D).

With respect to the sporocyst stage of flukes infected by *Urosporidium* sp, as mentioned above, Sapsido was the only clam bed where hyperparasitism was found in both digenetic sporocysts and metacercariae stages. However, there was only one clam individual seen to be parasitized by digenetic sporocysts having *Urosporidium* hyperparasitism.

In this specimen, histological observation showed a severe, destructive impact caused by the encroachment of cercaria-harbouring sporocysts on whole clam body. Apart from the area of the digestive gland where there were a few sporocysts residing, the other organs appeared to be displaced and compressed by the established network of sacs of cercariae and germ balls at different developmental periods.

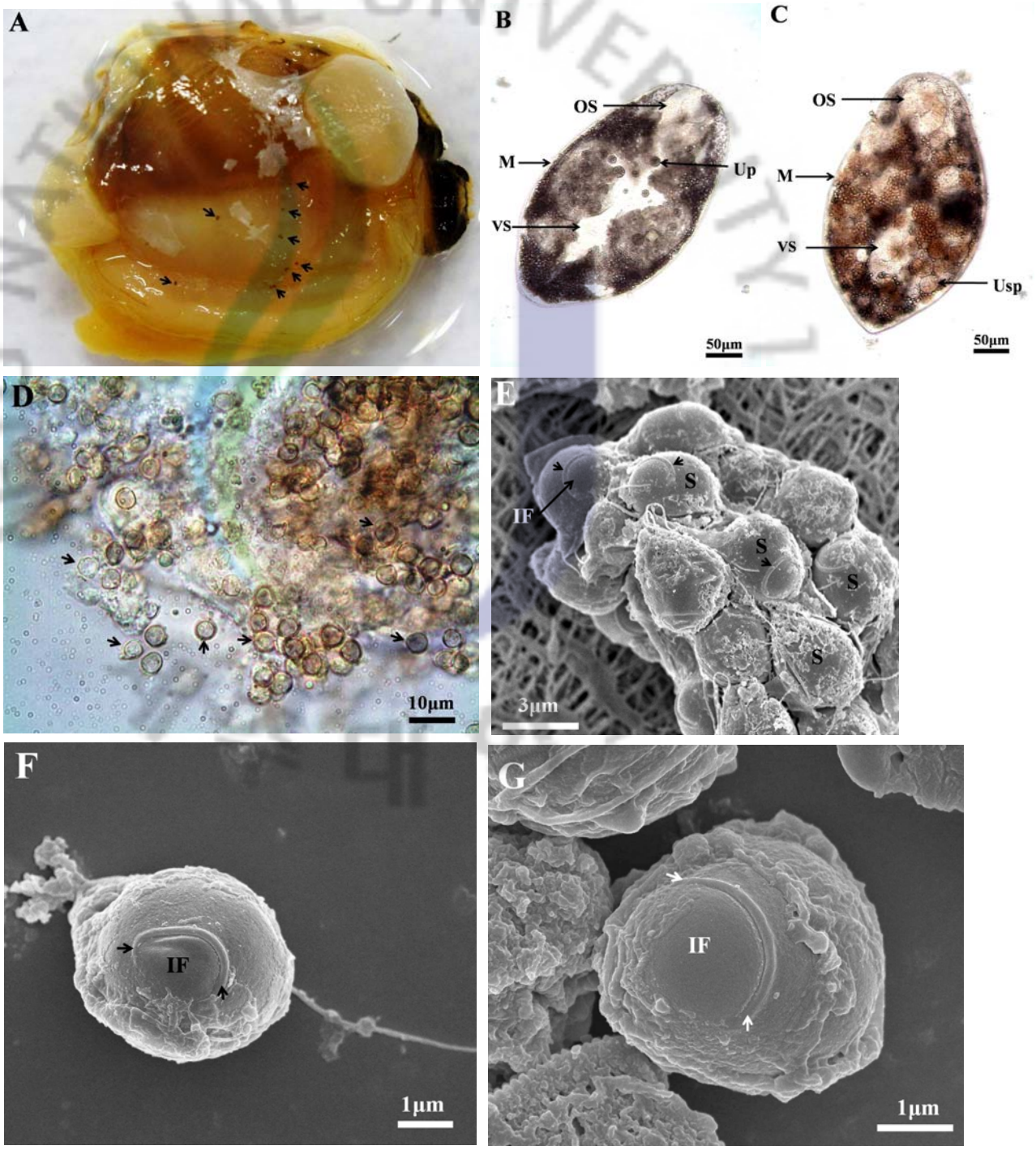

Fig.6. Light and electron micrographs showing *Urosporidium*. sp. in trematode parasite of Manila clam. A: clam containing trematode metacercariae infected by *Urosporidium*. sp (arrows). B: Metacercaria (M) infected by the plasmodia of *Urosporidium*. sp (UP); OS-oral sucker, VS-ventral sucker. C: Metacercaria (M) infected by the sporocysts of *Urosporidium*. sp (US). D: Some free and mature spores of *Urosporidium*. sp (arrows). showing spherical shapes with yellowish envelopes. E: A group of spores (S) observed by SEM, showing an orifice of the spore (arrows) is covered by an internal flap (IF) of wall material. F&G: Spore showing structure of the spore orifice (arrows) and IF.

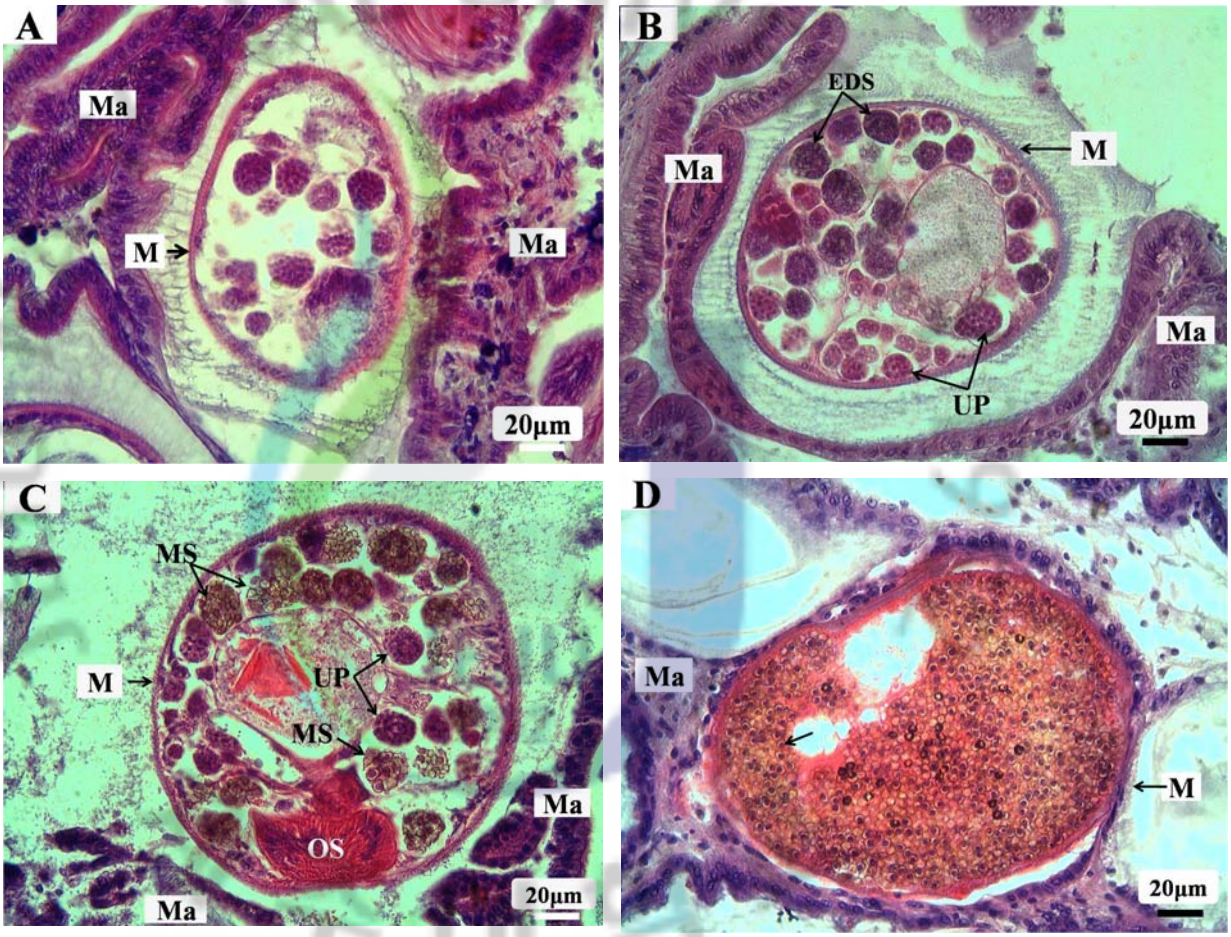

Fig.7. Histology showing Urosporidium hyperparasites inside the unencysted metacercaria embedded in mantle cavity of Manila clam. A: Multinucleate plasmodia (arrows) developing inside the metacercaria (M). B&C: Urosporidium sporocysts harboring numerous spores at various stages of early sporulation; plasmodia (UP) and early developing sporocyst (EDS) of *Urosporidium*.sp containing a number of immature spores. D: fully mature spores (arrows) in a destroyed metaeceracia. Ma: Mantle of clam; OS: oral sucker.

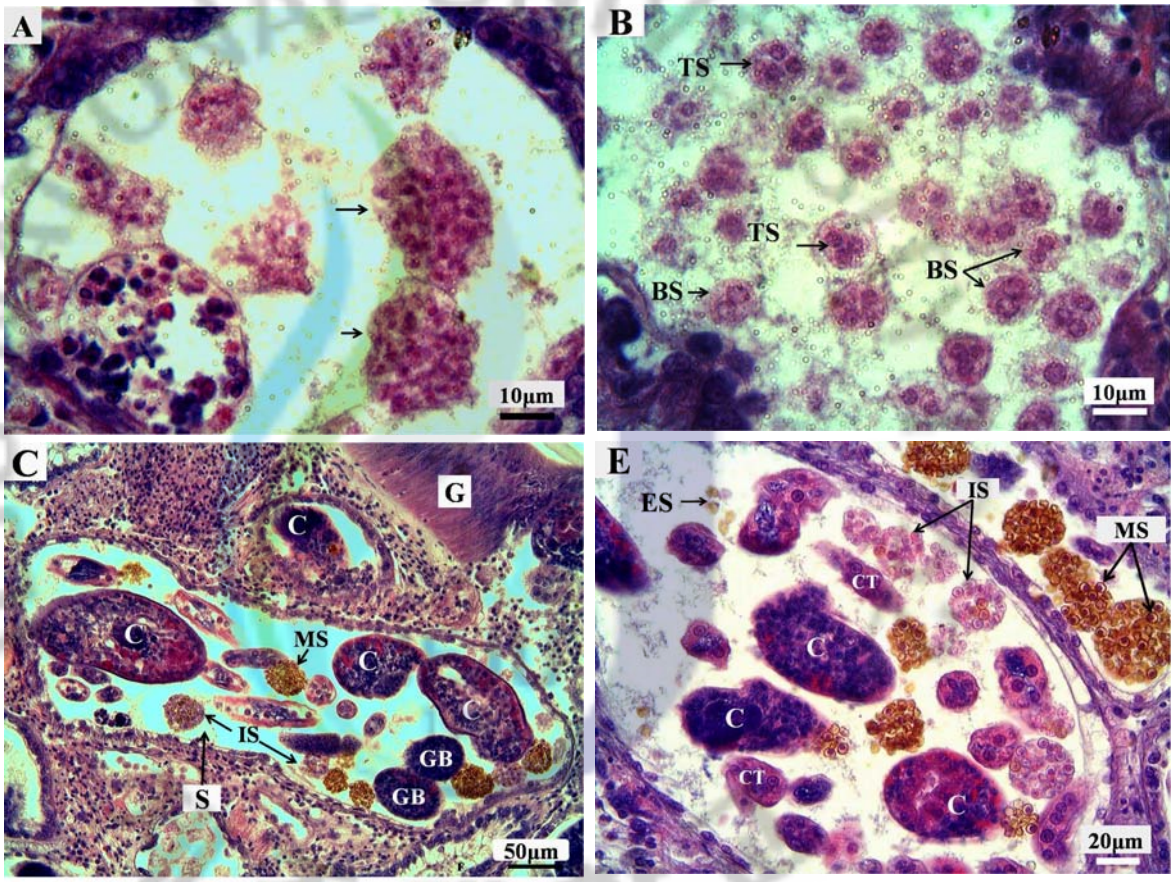

Fig.8. Histology showing different life stages of *Urosporidium*. sp inside trematode sporocysts embedded in the gonad of *R. philippinarum*. A: early multinucleate plasmodia (arrows). B: early sporogonic stages of *Urosporidium*. sp, note formation of binucleated sporonts (BS) and trinucleated sporonts (TS). C &D: Sporocyst of trematode (S) enclosing cercaria (C), tail of cercaria (CT), germinal balls (GB), inmature *Urosporidium* sporocysts (IS) and mature sporocysts (MS) ;ES: empty spore.

Table 5. Prevalence and infection intensity of trematode and *Urosporidium* .sp-infected metacercaria at the sampling sites. Mean, Min-Max: mean, minimum and maximum quantity of metacercariae found on a histological section.

Site	N	Trematode prevalence	*Urosporidium*.sp-infected metacercaria			
			Prevalence	Intensity		Stages of *Urosporidium*.sp
				Mean	Min-Max	
Seokchunri	40	40.0	2.5	2.0	0-2	Spore, sporocyst, plasmodia
Ojiri	40	27.5	5.0	3.0	2-4	Mature spore
Dokgotri	40	20.0	5.0	1.0	0-1	Spore, sporocyst, plasmodia
Naeri	40	25.0	5.0	1.0	0-1	Plasmodia
Gonam	40	35.0	22.5	2.0	1-5	Spore, sporocyst, plasmodia
Duiruini	30	43.3	2.5	1.0	0-1	Plasmodia, sporocyst
Hakseongri	40	20.0	2.5	1.0	0-1	Mature spore
Sapsido	40	17.5	5.0	2.0	0-2	Spore, sporocyst, plasmodia
Namdangri	25	48.0	24.0	1.5	1-2	Spore, sporocyst, plasmodia
Goheung	40	35.0	2.5	3.0	0-3	Spore, sporocyst, plasmodia

Table 6. Size (μm) of *Urosporidium*. sp found in the trematodes: all measurements are in length; SD: standard deviation. I: immature spore; M: mature spore; Min-Max: minimum and maximum length.

Site	Spore (μm)		N	Sporocyst (μm)		N	Plasmodia (μm)		N	Stage
	Mean-SD	Min-Max		Mean-SD	Min-Max		Mean-SD	Min-Max		of spore
Seokchunri	4.59 ± 0.34	4.01 - 5.15	14				16.54 ± 1.22	14.08 - 19.45	8	M
Ojinri	4.29 ± 0.35	3.57 - 5.53	317							M
Dogot	4.43 ± 0.36	3.73 - 5.49	40	19.71 ± 3.57	14.80 - 27.46	12	15.48 ± 3.40	12.30 - 19.55	6	M
Naeri							15.55 ± 1.64	12.45 - 21.69	10	
Gonam	4.24 ± 0.29	3.33 - 6.32	115	18.2 ± 3.71	11.27 - 26.24		16.64 ± 2.35	9.53 - 26.74	87	I, M
Deurini				26.98 ± 2.50	23.99 - 30.03	5	24.78 ± 5.42	19.94 - 30.64	3	I, M
Hakseongri	4.27 ± 0.31	3.63 - 4.82	30							M
Sapsido	5.19 ± 0.37	3.94 - 6.18	130	29.75 ± 6.58	17.54 - 46.72	58	24.67 ± 4.69	17.69 - 30.09	7	I, M
Namdangri	4.7 ± 0.38	3.40 - 5.68	58	24.04 ± 4.02	14.40 - 27.53	52	16.09 ± 2.78	11.93 - 23.71	13	I, M
Goheung	3.92 ± 0.29	3.02 - 4.82	53	16.12 ± 3.38	8.05 - 23.57	19	11.99 ± 2.09	9.34 - 11.61	10	I, M

A few sporocysts of *Urosporidium* hyperparasites, whose common shape was spherical to round, were found scattered within several fluke sacs located mostly in the gonadal tissues. The *Urosporidium* sp. showed many affinities with those described in the affected metacercariae: basophilic multinucleate plasmodia, and goldenish round sporocysts containing both empty spores and spores with endosporoplasm. Interestingly, a great number of developmental stages of *Urosporidium* cell division, including asexual reproduction (schizogony) and sporulation, were seen in trematode sporocyst lumens. The developing multinucleate cells (pre-plasmodia), plasmodia, and sporonts of *Urosporidium* sp. (intermediate developmental stages from plasmodia to sporocysts) were eosinophilic, and varied in shape and size (Fig 7A&B). Especially, as observed, only such multinucleated cells, and sometimes sporocysts, existed within trematode sacs without cercariae as well as germinal balls (Fig 7). It was also a fact that, although the density of digenetic sporocyst sacs was very dense in the infected clams, there were a few cercariae and germinal balls concurrently enclosing each sporocyst, particularly for sporocyst sacs infected by the hyperparasites. Conversely, two metacercariae found to be encysting in the foot tissue of a specimen did not displace any hyperparasite.

The number and size of *Urosporidium* spores within each infected trematode sporocyst parasitizing the Ojinri clams were slightly smaller than those from infected trematode metacercaria that were found in clams from the other beds.

During the present study, the hyperparasite prevalence varied among ten infected clam populations; a maximum level of 24 % infected clams was found in Namdangri, followed by 22.5% prevalence from the Gonam clams. The eight other sampling sites showed a relatively low prevalence, varying between 2.5-5% of total specimens examined per site.

3.4. Histochemical reaction of hyperparasite

Table 6 showed the number of hyperparasitized specimens from four sampling sites with three selected histochemical methods successfully applied. Most of the stained specimens exhibited a positive reaction with the stains used.

For acid fastness, the mature hyperparasites exhibited a characteristic staining reaction

with the Ziehl-Neelsen carbol fuchsin method as modified by Farley (1965). Acid-fast-positive was found in the mature spores whose sporoplasm stained profoundly luminous red, making their presence more visible in the metacercarial cavity. Conversely, the sporocysts of immature spores showed only a weak positivity; in particular, plasmodia and metacercaria tissues were completely unstained. It was also observed that many spores seemed to be mature due to the appearance of an very dark internal endosporoplasm, but did not stain with Ziehl-Neelsen's stain (Fig 8). This evidence indicates that acid fastness was created or present only in fully mature individuals of the *Urosporidium* spores.

PAS-reaction revealed the presence of carbohydrates in most of the components of the life cycle stages of only. Also, a small portion of tissue from some specific organs, and all metacercaiae, including *Urosporidium*-infected and noninfected individuals, also stained positively with PAS. With the pink-stained surface, the plasmodial wall and sporocyst wall containing either immature or mature spores were positive to PAS, but the color intensity of the former was much stronger (Fig 9). Especially, as described previously, the plasmodia observed herein were usually attached to metacercarial connective tissues, but *Urosporidium* sporocysts were mostly non-attaching within infected metacercariae. Moreover, while cytoplasm of these plasmodia was also weakly positive to PAS, only a few immature spores enclosed in the sporocysts became positive. Also, within the mostly destroyed metacercaria bodies, only immature spores were observed to stain slightly with PAS-stains, suggesting that carbohydrates had a negative relationship with the sporulation of *Urosporidium*.

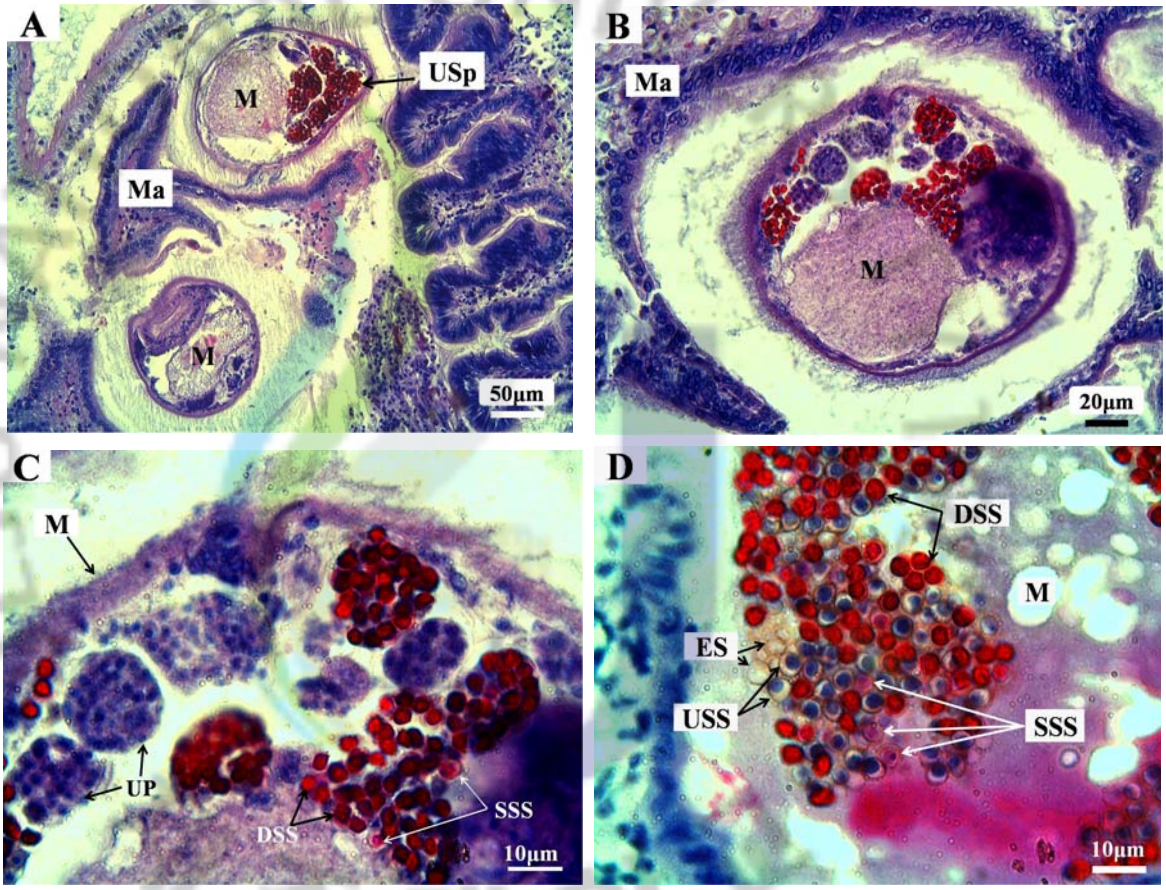

Fig.9. *Urosporidium* sp. stained with Ziehl-Neelsen carbol fuchsin method. A&B: Only metacercaria (M) invaded by *Urosporidium* sp. (USp) stained in red while metacercarial body and clam tissue were unstained with carbol fuchsin; C: unstained plasmodia (UP) and slightly stained spores (SSS) and dramatically stained spores (DSS) within stained Urosporidium sporocyst; D: a various maturity of Urosporidium spores.

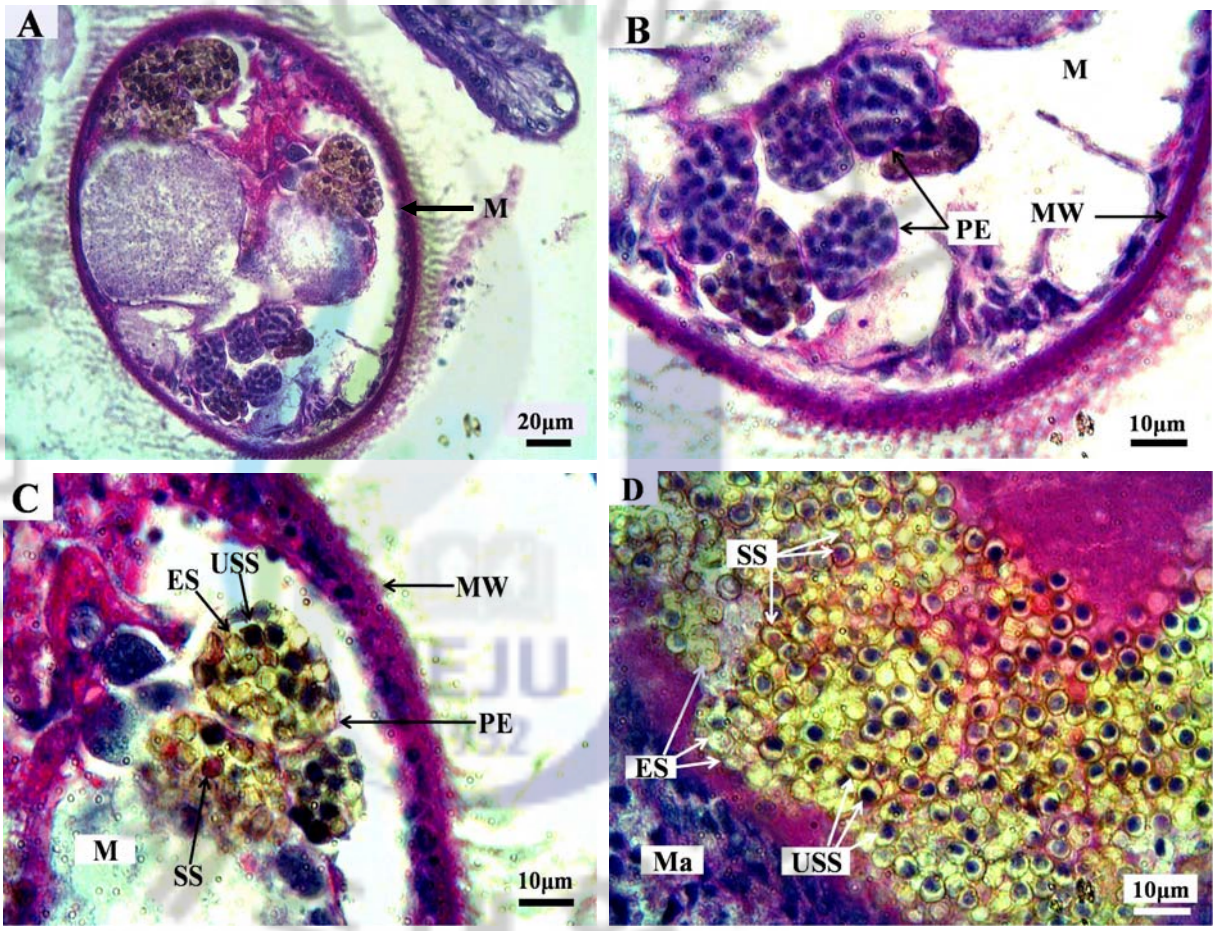

Fig.10. *Urosporidium* sp. stained with PAS (the periodic acid-Shiff test). A: whole body of metacercaria containing *Urosporidium* sp. stained positively with PAS. B: Higher magnification of the *Urosporidium* plasmodia and metacercaria wall stained in pink by PAS. C: envelope of *Urosporidium* sporocysts stained by PAS: D Fully mature *Urosporidium* spores show various color intensities to PAS;

(M: metacercaria; ES: empty spores; DSS: dramatically stained spore; MW: metacercarial wall; PE: plasmodial envelope; SSS: slightly stained spore; USS: unstained spore.

IV. DISCUSSION

4.1. Trematode infestation

In the present study, results from histological examination provided an overall guide concerning the status of trematode infestation of *Ruditapes philippinarum* populations in Korea, particularly on the west coast. An important consideration is that specimens at all locations surveyed were found to be infected by parasites, and this infection was encountered at all three types of clam beds. None of the intramolluscan developmental larval stages of trematodes were absent in clam bodies. Moreover, almost all organs appeared to be habitats for digenean larval development, the gonad and mantle cavity being the most suitable locations. It is therefore obvious that trematode-caused disease has become epidemic in *R. philippinarum*, at least during the spring.

The susceptibility of this species to a number of trematode larval species has been documented worldwide. Based on experimental infection and field observation on trematode infestation in *R. philippinarum*, a total of six digenetic families comprising more than seventeen species were detected parasitizing various locations in the host bodies (Table 7). In Korea, three out of all reported digenean species have also been reported infecting clams on both the south and west coasts. In this study, although identification of trematode species was not intended for all the clams infected, the distinct shapes of trematodes found in the clam populations revealed that co-infection of different trematode species can be the reason for the high trematode prevalence recorded herein.

As regards each type of larval trematode in *R. philippinarum*, histology demonstrated that metacercaria was the most common larval stage of trematodes, which were encountered in all study areas and exhibited a high mean prevalence of 27.9 % (299 specimens affected) and a mean intensity of 3.01 metacercariae per host. Brower *et al.*. (1992) also surveyed histologically the prevalence of trematode metacercariae in *R. philippinarum* from 21 localities in British Columbia, and the mean percentage of infected clams was 6.3 %, much lower than the level obtained in this study. In France, Lassalle *et al.* (2007) examined five natural clam beds along the French Atlantic coast. The data

showed that those clam populations were affected with a relatively high mean metacercarial prevalence of 39 % while the average metacercarial burden was one only live metacercaria per clam host. Regarding *R. philippinarum* communities in Korea, the infection level with metacercariae reported here was consistent with previous research. In the southern area of the country, Sohn *et al*, (1996) surveyed trematode infestation of coastal clams, and documented an intense infection condition by live *Parvatrema* spp. metacercariae; twelve out of thirteen clam beds were shown to be parasitized with a high mean rate of prevalence and intensity, 77.4% and 54.7 cysts per clam respectively. Recently, Limpanont's histological study (2010) on the characteristics and composition of parasites in clams across the country also showed a similar wide distribution of this kind of parasite. However, the degree of infection amongst locations recorded was generally less severe than that recorded in this study; only eight clam beds revealed prevalence higher than 20 %, compared to nineteen sites herein.

With a 6.05 % mean prevalence, varying from 0 to 15 % among the study locations, the infestation of digenetic sporocysts in clam hosts demonstrateed in this histological survey was relatively low. The similarly low level of infection with trematode sporocysts was also described in previous studies in which seasonal changes of environmental temperature and the life-cycle of trematode species were thought to have a positive relationship with a great variation of sporocyst infection rates (Pike, 1968; Shimura and Kuwabara, 1984). In Korea, during a one-year survey on cercarial infestation of clam beds in Seo-myon and Seo-chon gun, Kim and Chun (1983) demonstrated the fact that while infection with developing cercariae showed a peak of 5.74% in March, no infected clams were found in May and June. The same pattern of cercarial infestation was also reported from clam populations in Jeju-do (the southernmost island of Korea) which showed a monthly variation of cercaria prevalence from 0 to 12% in the clams. In comparison with these studies above, the absence of cercariae at many clam beds in this study was supposedly due to the unavailability of intermediate or definitive hosts of trematodes, such as gastropods and sea birds, such as those mentioned by Lauckner (1984) and Chung *et al.* (2007; 2010).

Table 7. Various species of trematodes in *R. philippinarum* distributed worldwide. C-cercaria; M-metacercaria; S-sporocysts), method (method of trematode identification: MC-morphology characteristics; EI-experimental infestation; MA-molercular identification)

Species	Family	Larval stage	Location	Method	References
Himasthla quissetensis	Echinostomatidae	C, M	America	EI	Cheng *et al.*, 1966
			Southwestern France	MC	Lassalle *et al.*, 2007
Cercaria tapidis	Echinostomatidae	S	West coast of Korea	MC	Kim & Chun, 1981&1983
			East coast of Japan	MC	Shimura and Kuwabara, 1984
Cercaria pectinata	Echinostomatidae	S	East coast of Japan	MC	Shimura and Kuwabara, 1984
Cercaria sp	Echinostomatidae	S	East coast of Japan	MC	Shimura and Kuwabara, 1984
Derogenes varicus	Hemiuridae	M	West coast of Canada	MC	Bower *et al.*, 1992
Parvatrema spp	Gymnophallidae	M	South & east coast of Korea	MC	Sohn *et al.*, 1996
Parvatrema duboisi	Gymnophallidae	S, C, M	Southwestern Japan	MC, EI, MI	Yanagida *et al.*, 2009
Himasthla alincia	Echinostomatidae	M	Southwestern coast of Korea	MC, EI	Han *et al.*, 2009
Himasthla continua	Echinostomatidae	M	Southwestern France	MC	de Montaudouin *et al.*, 2000
					Dang *et al.*, 2009
Himasthla elongata	Echinostomatidae	M	Southwestern France	MC	de Montaudouin *et al.*, 2000
					Lassalle *et al.*, 2007
		C, M	Southwestern France	MC	Dang *et al.*, 2009
Himasthla interrupta	Echinostomatidae	M	Southwestern France	MC	de Montaudouin *et al.*, 2000
					Lassalle *et al.*,2007
Meiogymnophallus fossarum	Gymnophallidae	M	Southwestern France	MC	de Montaudouin *et al.*, 2000
Psilostomum brevicole	Psilostomidae	M	Southwestern France	MC	de Montaudouin *et al.*, 2000
					Lassalle *et al.*,2007
					Dang *et al.*,2009
Renicola roscovita	Renicolidae	M	Southwestern France	MC	de Montaudouin *et al.*, 2000
Curtuteria arguinae	Echinostomatidae	M	Southwestern France	MC	Lassalle *et al.*, 2007
					Dang *et al.*, 2009
Renicola roscovita	Renicolidae	M	Southwestern France	MC	Dang *et al.*,2009

Histopathological demonstrations of infestation caused by metacercariae encysting in the musculature were more severe than by those attached to the mantle matrices of *R. philippinarum*. The majority of parasite-induced encystments stimulated a massive number of hemocytes surrounding the cysts, indicating probably an internal host defence reaction. The same phenomenon was recorded by Bower *et al.* (1992) as metacercaria was discovered embedded in the digestive gland of clams. Cheng *et al.* (1966), who observed the effects of *Himasthla alincia* on eight marine species of clams, oysters, and mussels infected with the cercarial stage, demonstrated that the difference between host species and their parasite-infected organs was in relation to the composition and intensity of the cellular encapsulation of the host. Furthermore, it was believed that the metacercaria-released enclosure, which was seen frequently in this study, was the stimulating factor that produced leucocytic response in *R. philippinarum* which was more severe than that in the other hosts (Cheng *et al.,1*966). Such intense hemocytic response of the host was not apparent in the mantle where greater numbers of metacercariae were found in this study.

According to Ching (1995), each species with unencysted metacercariae colonizes its own microhabitat on the mantle cavity of its hosts, and this location is associated with the severity of the host's reaction. Shell deterioration, depletion of host-body reserves, behavior changes, growth reduction, and mortality increase are pathological effects reported from various marine bivalves with metacercarial infection (Lauckner, 1983). In the present observation, histology demonstrated that degree of hyperplasia and parasitic erosion produced on the mantle appeared to be related to the abundance of non-encysted metacercariae. Furthermore, the host haemocytic infiltration was also commonly seen at the epithelial layer adjacent to the parasites. Silva *et al.*, (2009) hypothesized that the formation of haemocytic infiltration is the initial host's capacity, in stout razor clams, to isolate and then ruin the unencysted parasites. However, in this study, such metacercariae were rarely seen to be encapsulated by the connective tissue of the host mantle. For morphological change caused by metacercariae, Ituarte *et al.*,(2001) proved that infestation by Gymnophallidae and Lepocreadiidae metacercariae did result in a profound alteration of the shell-mantle complex and organic shell matrix

of *Gaimardia trapesina* clams. Due to parasite impact, the host mantle was stimulated to abnormally proliferate and produce a dome-shaped covering; concurrently the shells were also modified to form open, rounded shallow pits within the shell's inner surfaces. Such structures were found at the areas where the parasites could be living or had escaped from the host (Ituarte *et al.*, 2001). For bivalves, the mantle functions in the formation and growth of the shell, which protects the internal gills. Therefore, hyperplasia and multiple folds in this organ, which were observed in this study, may disturb the energy preservation and normal activity of the clams.

The level of parasitic castration in clam gonads due to germinal-ball sacs and sporocysts of trematodes appeared to be related to the degree of development of the larvae involved. In comparison between hosts whose gonads were invaded only by the first type of trematodes and those whose gonads were parasitized by both the first and second types, the density of the former was less than that of the latter. Also, the gonads of the former showed less damage than that of the latter. These phenomena were possibly due to the multiplication process of germinal sacs and the differentiation of their internal components that resulted in an enormous number of successive generations. As a result, their impact level on the infected hosts also increased. Lauckner (1983) deduced that trematode sporocysts tend to increase in quantity in the intermediate hosts before their metamorphosis to the next generation occurred. However, environmental conditions inside the host as well as the duration required for a germinal ball to become a cercaria is not well known, at least in case of the Manila clam.

The pathological impact produced by trematode sporocysts in marine bivalves has been well documented. According to Bower *et al.*, (1994) infestation with sporocysts and cercaria in species of the famililies Gymnophallidae and Bucephalidae was observed to cause castration, hermaphroditism, and protandry in *Crassostrea virginica*, or to interfere seriously with the formation of pearls in *Pinctada martensi*. Santos and Coimbra (1995) reported that infestation with *Proctoeces maculates* sporocysts restricted gonadal development of mussels, and this effect was gender-dependent, impacting particularly male hosts. Besides the marked reduction of host reproductive tissue, Silva *et al.*, (2002) provided some evidence showing that the immune system of *Perna perna* also decreased when infected

with *Bucephalus* sporocysts. In the clam *Eurhomalea lenticularis*, Valderrama *et al.*, (2004) found that Plagiorchidae sporocysts not only generated parasitic castration in the parasitized gonad but also host germ cells in areas that have no sprorocysts were deformed. These reports indicated that the intense parasitism of trematode sporocysts elicited a generalized impact on the entire body of the host, affecting reproductive ability and reducing host resistance to environmental stress. Regarding the histopathological findings in the sporocyst-infected clams reported here, the gradual destruction and replacement of host tissue, particularly gonads, by a massive invasion of parasites obviously affected host fecundity and possibly also increased clam vulnerability to other pathogenic agents. This is typical with the *Perkinsus* parasites which have been known to establish themselves locally in the areas examined (Limpanont, 2010)

4.2. Hyperparasite infestation

Marine hyperparasites are often referred to several microparasite species which have been found to infect various parasites of polychaetes, mollusks, and crustaceans (Sprague and Couch, 1971; Sprague, 1979). Among the three different phyla whose few species cause hyperparasitism, in the Haplosporidia, only the genus *Urosporidium* has been known to form endohyperparasites (Freeman, 2005). Members of the phylum Haplosporidia are obligate parasitic protists that produce thick-walled, uninucleated spores with an orifice at one spore pole covered by a characteristic lid. The ultramorphology of spore ornamentations and position of the lid, covering the internal or external spore orifice, is accepted as the best characteristics for distinguishing this phylum from the others, as well as for distinguishing species in this phylum (Burreson and Ford, 2004). The life cycle of none of the Haplosporidian species has been known, although most of the Haplosporidian life stages, including uninucleated cells, multinucleated plasmodia, and spores, are visually observed in the body of many species.

Of all parasites infecting the Manila clam, only trematodes were invaded by *Urosporidium* sp., probably indicating that trematodes were suitable or susceptible hosts for harboring hyperparasites. Out

of ten species in *Urosporidium* found to be parasitic in parasites of marine mollusks, six species were discovered to inhabit digenetic larvae. Furthermore, *Turbellaria*, one of the common macroparasites in marine bivalves, was reported to be invaded by two *Urosporidium* spp. hyperparasites, in a cockle and oysters (Anderson *et al.*,1993; Carballal *et al.*, 2005). However, in this study they did not show any hyperparasitism although they were present in the same clams that were infected by the *Urosporidium* sp. The high susceptibility of trematodes to hyperparasites may be related to their complex life span in which free-living larval stages have many possibilities to contact potential hyperparasitic pathogens. According to Freeman (2005), Udonellid ectohyperparasites have been reported only from caligid copepods and argulids, which are known to swim freely in the water column as adults in order to find new fish hosts, but have not been found on parasitic copepods with sessile non-swimming adult stages.

The direct trematode host, *Urosporidium hyperparasitism* is destructive to infected metacercariae but causes a relative reduction in the number of cercariae and germinal balls produced within infected digenetic sporocyst sacs. From the current observevations, nonencysted metacercariae were the only free-living larval stage of trematode attacked by *Urosporidium* sp. These results were different from those of previous publications which revealed that the hosts of the hyperparasites were involved both free-swimming and encysted metacercariae. However, the general impact of these microhyperparasites on their hosts was similar. The *Urosporidium* sp. proliferate rapidly and generate an abundant number of successive generations in the gradually enlarged host body. As a result, the infected organs of the host are destroyed and the release of the generated hyperparasite spores cause host destruction (Couch, 1974; Shields and Overstreet, 2007). Similarly, in several marine bivalves, the deleterious effects of hyperparasites on trematode sporocysts concurrently residing in their indirect hosts (bivalves) have been recorded. According to Lauckner (1983), in the majority of hyperparasite-affected digenetic sporocysts, the encroachment and establishment of *Urosporidium* sp takes place both inside the sporocyst wall and the lumen. Concurrently a complete destruction of all cercariae in each infected sporocyst sac also occurs. In other cases, similar to the current study, no developmental stages of hyperparasites were observed in the trematode sporocyst wall, and it was revealed that sporocysts

and cercariae are not always involved, as mentioned by Lauckner (1983). However, in this study, the decline in number of sporocyst-generated products (cercariae and germinal balls) and their disappearance happened in the presence of proliferating hyperparasites. This could show that reproduction of sporocysts was significantly impacted, and that the sporocysts were utilized as a culture medium and transmission means for *Urosporidium* generations.

Infestation with *Urosporidium* sp caused destructive impact on the trematodes but with no effect on the clams. Thus, although trematode individuals, including metacercariae and sporocysts, hyperparasitized by *Urosporidium* sp at premature stages appeared healthy and were still able to attach to, or even damage, the clam tissues, no abnormally pathogenic condition resulted from the presence of these trematodes in the clam hosts in comparison with that caused by uninfected trematodes. Similar descriptions of such hyperparasite-parasite-host interaction has been reported in digenetic sporocysts metacercaiae parasitizing clams, crabs (Perkins, 1971; 1975; Ormières *et al.*, 1973; Couch, 1974), turbellarians in oysters and cockles (Anderson *et al.*, 1993; Carballal *et al.*, 2005). However, negative effects appear considerably since hyperparasites sporulate. As a result, the infected parasites become dark and highly visible as “pepper-spots” against the light-colored tissue of the host. The hosts with such a hyperparasite-parasite complex are either unmarketable or have a lesser value, and therefore negatively influence the seafood industry (Perkins, 1979; Shields and Overstreet, 2007).

Concerning the histochemical components of *Urosporidium* sp., the positive reaction of their mature spore sporoplasm with carbol fuchsin staining can confirm the fact that these hyperparasites form mature spores inside their trematode hosts and these spores have acid fastness. For Haplosporidia, the acid fastness has been detected for the first time in a comparative experiment in which Farley (1965) modified Ziehl-Neelsen carbol fuchsin technique in order to distinguish two pathogenic agents of oysters; SSO-causing *Minchinia costalis* and MSX-causing *Minchinia nelsoni*. Only mature spores of SSO did show the positive reaction and, as a consequence, this technique has been recommended as a detection method for the presence of *Minchinia costalis* in infected oysters. In later years, however, acid fast mature spores have also been documented from *Haplosporidium tumefacientis* parasitizing

mussels (Taylor, 1966), *H. malacobdellae* in Hoplonemertean worms (Varndell, 1981a) and *Haplosporidium sp.* in rock oysters (Hine and Thorne, 2002). The nature of acid-fastness in Haplosporidia has remained unknown to date, but Farley (1965) supposed that it is different from bacteria whose acid fastness is due to a waxy sheath. He believed that the positivity of Haplosporidia sporoplasm to Ziehl-Neelsen carbol fuchsin derived from a chemical bond created between the stain and an oxidized lipoid in the sporoplasm. In addition to Farley's assumption, Varndell (1981b) mentioned that the acid-fast substance of mature spores was possibly a mechanism or an enzyme-mediated process involved in the production of the spore wall.

For the periodic acid-Shiff test (PAS test), the gradual decrease and disappearance in color intensity of positive reactions seen in different types of *Urosporidium* sp. showed a variation of carbohydrate material throughout *Urosporidium* sp. sporogenesis. Similar to the acid fastness, the carbohydrate role in the sporulation processes of Haplosporidia has not been fully known, although some research has demonstrated its existence in association with Haplosporidia growth. Studying the histochemical reaction of *H. tumefacientis*, Taylor (1966) discovered that, while the cytoplasm of sporocysts and immature spores appeared mostly PAS-negative, that of mature spores and the operculum of such mature spores were strongly PAS-positive. The author also reported that the presence of neutral mucopolysaccharides, muco- or glycoproteins, or a complex of them produce the PAS positivity instead of the glycogen. However, different from the result reported by Taylor (1966), Varndell (1980) reported that the PAS-positive response became reduced in intensity as *H. malacobdellae* sporogony proceeded. Particularly, Varndell (1981a) proved that such a decrease correlated with the appearance and increase in activity of three carbohydrase enzymes at early sporogony stage. Therefore, the produced short-chain carbohydrates can participate in the production of chemical energy in the parasite. In the current study, the entire metacercarial body, particularly its connective tissues, stained strongly positive with PAS, and *Urosporidium* sp. plasmodia were found to attach to these locations, while hyperparasite sporocysts mostly detached, possibly demonstrating an hyperparasite-metacercaria interaction in term of carbohydrate energy. Apparently, in such interaction

metacercaria are a reservoir of carbohydrate material available to hyperparasites, and the sequentially destroyed bodies of metacerca hosts could be an independent site of hyperparasites on this energy resource.

The *Urosporidium* life cycle as well as how the hyperparasites successfully invade the digenetic parasites of Manila clam are unknown. For Haplosporidia species, attempts to identify their life cycle and mode of infection have failed but amoebulae, an organism hatched from Haplosporidia spores, has been believed to be the infective stage (Azevedo, 1985; Lauckner, 1983). In addition, the assumption that there is the existence of another host whose role is serving as reservoir or intermediate host has remained unproven (Burreson and Ford, 2004). In this histological survey, a large number of trematodes in clams were hyperparasited by various *Urosprodium* stages but there were no *Urosprodium*-like forms seen in the clams and cercariae. Moreover, developing *Urosporidium* sp. cells at both schizogony and sporulation processes were found in cercaria-containing sporocysts, but only the sporulation observed in the metacercariae. These observations possibly reveal that infection by *Urosporidium* hyperparasites on trematodes likely occurred in the miracidium stage, and cercarial generations reproduced from such miracidum could carry a certain form of the pathogen. Thus, this potential pathogen could develop either directly in infected cercariae or after its host became metacercaria.

V.CONCLUSION

Trematode parasite has been well established and become a severe epizootic in the commercial clam beds in Korean coastal waters. In April, the clams surveyed were mostly in early and late developing states of the gametogenic process. They were mainly parasitized by metacercaria and cercaria-containing sporocyst of unidentified trematode species, causing various impacts on the host organs.

Epithelial hyperplasia of the mantle, replacement of foot tissue by parasitic cysts, follicular castration of the gonad were the most common histopathological exhibitions observed in the clams hosting the trematode larva. These tokens indicated that the infection with trematode larva not only deformed the normal body structure, restraining the reproductive ability of the hosts, but possibly also impacting on longevity and resistance of the infected clams to environmental conditions.

The appearance of *Urosporidium* sp. found for the first time in this study, widely hyperparasitizing in populations of Manila clam appeared as a biological control factor to domination of the trematode parasite. Along with sporulation of *Urosporidium* sp. inside the body of trematodes was gradual destruction of these hosts, but no histopathological sign relating to hyperparasitism to the clams recorded.

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