Mass mortality of pearl oyster (*Pinctada fucata* (Gould)) in Japan in 2019 and 2020 is caused by an unidentified infectious agent

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Mass mortality of 0-year-old pearl oysters, *Pinctada fucata* (Gould), and anomalies in adults were observed in Japan's major pearl farming areas in the summer of 2019 and 2020. Although adult oyster mortality was low, both adult and juvenile oysters underwent atrophy of the soft body, detachment of the mantle from nacre (the shiny inner surface of the valves), deposition of brownish material on the nacre, and loss of nacre luster. Infection trials were conducted to verify the involvement of pathogens in this phenomenon. Healthy adult pearl oysters were obtained from areas where this disease had not occurred to use as the recipients. The sources of infection were either affected adult oysters with atrophied soft bodies or batches of juveniles in which mortality had reached conspicuous levels. Transmission of the disease to the healthy oysters were tested either by cohabitation with affected oysters or by injections of the hemolymph of affected animals. The injection infection test examined the effects of filtration and chloroform exposure on the pathogen. Occurrence of the disease was confirmed by the appearance of brown deposits on the nacre and loss of nacre luster. The abnormalities of nacre were clearly reproduced in recipient shells in 3 out of 4 cohabitation trials with affected oysters. The disease was also reproduced in 6 out of 6 injection trails either with hemolymph filtered through 100 nm filter or with hemolymph treated with chloroform. In a serial passage with hemolymph injections, the disease was successfully transmitted through 8 passages. These results suggest that the etiology of the disease is a non-enveloped virus with a diameter ≤100 nm.

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Abstract

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17 Japan's major pearl farming areas in the summer of 2019 and 2020. Although adult oyster mortality was low, both

18 adult and juvenile oysters underwent atrophy of the soft body, detachment of the mantle from nacre (the shiny inner

19 surface of the valves), deposition of brownish material on the nacre, and loss of nacre luster. Infection trials were

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23 Transmission of the disease to the healthy oysters were tested either by cohabitation with affected oysters or by

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26 deposits on the nacre and loss of nacre luster. The abnormalities of nacre were clearly reproduced in recipient shells

27 in 3 out of 4 cohabitation trials with affected oysters. The disease was also reproduced in 6 out of 6 injection trails

28 either with hemolymph filtered through 100 nm filter or with hemolymph treated with chloroform. In a serial

29 passage with hemolymph injections, the disease was successfully transmitted through 8 passages. These results

30 suggest that the etiology of the disease is a non-enveloped virus with a diameter ≤100 nm.

Introduction

33   The Akoya pearl oyster, *Pinctada fucata* (Gould), is the major species that is used for pearl culture in Japan.

34 In recent years, the annual production of pearls in Japan has been approximately 20 tons and the annual export value

35 is approximately 30 billion yen, which is second only to scallops. More than 90% of Akoya pearls are produced in

36 Ehime, Nagasaki, and Mie prefectures (Fig. 1).

37   Mass mortalities of juvenile Akoya oysters and adult Akoya oysters with atrophy of the soft body were

38 reported on pearl oyster farms in Mie Prefecture in June 2019 and Ehime Prefecture in early August 2019. Later in

39 2019, similar cases were reported from major pearl production areas, such as Nagasaki, Kumamoto, and Oita

40 prefectures. In particular, the death of 0-year-old oysters (hereinafter referred to as juveniles) was considerable.

41 According to the inquiry conducted by the Mie Prefecture Fisheries Research Institute to multiple pearl farming

42 companies in three regions of the prefecture, the mortalities of juveniles and adults were 56-74% and 23–24%,

43 respectively in 2019, whereas those in typical years were 15% and 9–16%. In 2020, similar abnormalities occurred

44 in all major pearl farming regions in the country, from June to November when the water temperature was high,

45 attaining the peak in June or July.

46   To date, the major infectious diseases known to affect *P. fucata* oysters in Japan are Akoya oyster disease

47 (Kurokawa et al., 1999, Matsuyama et al., 2017, 2018, 2019), black-spot shell disease (Sakatoku et al., 2018),

48 marine birnavirus infection (Suzuki et al. 1998a, b), and trematode infestation (Sakaguchi et al., 1968). However, the

49 mortality or abnormalities that occurred in 2019 and 2020 differ from any of these diseases and are referred to herein

50 as "summer atrophy". In healthy pearl oysters, the outer edge of the mantle is in close contact with the edge of the

51 nacre surface (Fig. 2D). In the oysters with summer atrophy, the mantle was detached from the nacre, and the entire

52 soft body contracts toward the dorsal margin (Fig. 2A). Shell spines often developed in the infected group (Fig. 2C),

53 and thus, the shell formation is probably normal until just before the disease onset. While the pearl oyster is

54 sometimes atrophied due to low water temperatures in winter, no disease accompanied by atrophy during the period

55 of high water temperatures has been reported until the occurrence of summer atrophy. Pearl oysters that have

56 recovered from summer atrophy showed symptoms of so-called "shell disease" (Huang et al., 2019), which is

57 characterized by brownish deposit on the nacre and loss of nacre luster often in the areas close to the edge of the

58 nacre (Fig. 2A–C). It is speculated that the deposition of brownish substance occurs due to the formation of a

59 prismatic layer on the surface of the nacre during the process of the exfoliated mantle returns to its original position

60 (Sano et al., 2021). The prismatic layer is usually formed on the outer layer of the nacre. The loss of luster is

61 probably the result of denaturation of the nacre by being exposed to seawater when the mantle is detached from the

62 nacre surface. The abnormalities in the nacre were observed in 60-97% of the oysters that survived summer atrophy,

63 according to a survey by the Mie Prefecture Fisheries Research Institute. Black-spot shell disease also causes

64 aberrant pigmentation in the nacre, but the deposits are darker than those occurring with summer atrophy.

65 Furthermore, deposition of black-spot shell disease typically occurs towards the dorsal edge (Fig. 2E), and death

66 does not occur. Akoya oyster disease causes atrophy of the soft body, whereas the nacre remains normal (Fig. 2F),

67 and mortality is high among adults (Iwanaga et al., 2008). Although mortality from marine birnavirus infection have

68 been reported, the pathogenicity of the virus is relatively low (Suzuki et al., 1998b).

69   Environmental factors have been suspected of the cause of the disease, such as high water temperatures,

70 decrease in food plankton, or chemical pollution. However, it is unlikely that these environmental changes caused

71 summer atrophy, considering that the disease with the same symptoms occurred almost simultaneously in most of

72   the pearl culture regions throughout Japan, where environmental conditions vary substantially. In addition, no

73   abnormalities have been reported in organisms other than pearl oysters, such as other shellfishes and fish, in the

74   areas where summer atrophy has been reported. On the other hand, the disease could be explained by an infectious

75   agent, which might have spread in the first year from the original outbreak through the active transportation of

76   juvenile oysters among culture areas, causing outbreaks almost simultaneously at a variety of locations across the

77   country in the second year. In this study, we conducted infection tests to verify whether summer atrophy is a

78   transmissible disease.

79

80   ## Materials & Methods

81   **Animals**

82   Details and localities of the pearl oysters, *Pinctada fucata* (Gould), used in each infection test are shown in

83   Table 1 and Fig. 1. One-year-old, healthy pearl oysters were obtained from Ishikawa or Kanagawa Prefecture, where

84   pearls are not cultivated (Fig. 1, Table 1). These oysters were used as recipients and negative control donors in the

85   infection tests. The pearl oysters from Ishikawa Prefecture that were used as recipients in Test 2 showed slight

86   pigmentation on the nacre in 2 of the 10 individuals observed, although the nature of pigmentation was much darker

87   than those typically found in the oysters affected by summer atrophy and rather similar to that of black-spot shell

88   disease. For the donors, shells exhibiting atrophy in the soft body were obtained from a pearl oyster farmer in Mie

89   Prefecture (Table 1). Prior to the start of each experiment, 5 or 10 individuals were randomly sampled from each

90   batch to examine atrophy of the soft body and nacre abnormality. Oysters of each experimental group were reared in

91   the same 65-L tank and different groups were separately reared in different 65-L tanks. The tanks were filled with

92   56 L running seawater (approximately 250 mL/min) filtered with a 1-μm pore size filter and maintained at 23–25°C

93   under the natural photoperiod in the laboratory. For food, 500 mL of cultured diatoms (*Chaetoceros neogracile*,

94   approximately 5×10^5 cells/mL), was added to each aquarium five times per week.

95

96   **Scoring degree of disease**

97   Although the infection tests were performed using 1-year-old adult oysters in this study, the mortality by

98   summer atrophy of shells over 1-year-old is low. Consequently, severity of the disease in the infection tests could

99   not be assessed by mortality. The atrophy of the soft body is characteristic of the disease. However, the atrophy

100   occurs only transiently at different times in different individual oysters, and shells must be opened when the

101   atrophy is most severe, to determine the severity of the disease. This is intrinsically difficult. On the other hand,

102   observations of the valves of spontaneously diseased oysters suggest that the deposition of brown pigment and loss

103   of nacre luster are also characteristic of this disease. These nacre abnormalities remain long after the oysters

104   recover from the disease, so that the shells can be opened at any arbitrary timing to determine the severity of

105   the disease. Therefore, in this study, the degree of disease severity was determined using abnormalities in the nacre.

106   The extent of abnormalities in the nacre was quantified with a subjective index as shown in Fig. 3. For the

107   infection tests, the scores for the two valves of an individual were totaled for each pearl oyster, and the sum of the

108   scores of oysters in one group was divided by the number of tested oysters in the group to obtain the mean shell

109   score of the group. For the investigation of the nacre of farmed oysters that was affected by spontaneously occurring

110   summer atrophy, the sum of the scores of valves for each group of oysters was divided by the number of observed

111   valves to obtain the mean shell score of the group. This is because the two valves of a stored individual oyster had

112   usually fallen apart and reconstruction of an individual by two valves was difficult.

113

114   **Investigation of spontaneously occurring disease in farmed oysters**

115   Atrophy of the soft body and abnormalities in the nacreous layer were investigated in cultured and wild pearl

116   oysters. Cultured oysters were obtained from the areas where summer atrophy is occurring, and wild, disease-free

117   oysters were from the areas where the disease has not been reported (Table 2). We also observed the nacreous layer

118   of stored valves of cultured pearl oysters. These oysters had been collected in Mie prefecture before June 2019, prior

119   to the first outbreak of summer atrophy.

120

121   **Test 1: Cohabitation and injection challenge using adult pearl oysters as the infection source**

122   In the cohabitation test, 5 diseased 3-year-old oysters and 14 healthy oysters were reared in different net cages

123   set in a 65-L tank under the conditions described above so that the diseased and healthy oysters are not in contact

124   with each other. For the negative control, 19 healthy oysters were reared similarly in another 60-L tank. In the

125   injection test, the hemolymph of 15 diseased oysters was collected from the adductor muscles. The hemolymph was

126   pooled and centrifuged at 1200 $\times g$ for 10 min to obtain the supernatant, which was used as the inoculum. This

127   inoculum was injected into the adductor muscles of 14 healthy pearl oysters (100 μl/individual) with 23 × 1/4 gauge

128   needles. For the negative control, 14 healthy oysters were inoculated with hemolymph into the adductor muscles

129   using supernatant prepared from 3 healthy oysters by the above method. Experiments were performed at two

130   different water temperatures, 25°C and 22–23°C. That is, a total of eight test groups were set (Table 2). The

131   mortality was monitored during the experiment. Since the abnormality on the nacre seemed to take several days to

132   appear and mortality sometimes occurred early in the experiments, the shell scores were determined only on

133   surviving specimens 27 days after the start of each experiment.

134

135   **Test 2: Injection challenge using juvenile pearl oysters as the infection source**

136   Soft bodies were collected from 12 0-year-old juveniles from an affected group. They were pooled (0.41 g in

137   total wet weight), chopped with scissors in 2 mL of autoclaved seawater, and centrifuged at 1200 $\times g$ for 10 min to

138   obtain the supernatant as the inoculum. The inoculum for the negative control was prepared in the same way from

139   healthy pearl oysters (0.43 g in total wet weight). Each inoculum was injected into the adductor muscles of 10

140   healthy oysters (100 μl/individual). The mortality was monitored, and the shell scores were determined on surviving

141   individuals 18 days after the injections.

142

143   **Test 3: Filterability**

144   Soft bodies were collected from 20 diseased 0-year-old juveniles, pooled (4.9 g in total wet weight), and cut

145   into small pieces with scissors. The volume of the minced tissues was adjusted to 50 mL with autoclaved seawater

146   and centrifuged at 1200 ×g for 10 min to obtain the supernatant. The supernatant was then sequentially filtered

147   through 0.8 μm, 0.45 μm, 0.22 μm, and 0.1 μm filters (Millipore, Millipore Co., Tokyo, Japan). Part of each filtrate

148   was saved and inoculated to the adductor muscles of 7 healthy oysters (100 μl/individual). Thus, the total of 28

149   shells were used. For the positive control, diseased oysters (N = 5) and healthy oysters (N = 7) were reared in the

150   same aquarium as in Test 1, since it had been known that the disease could be transmitted through cohabitation by

151   the time when this test was conducted. Two negative control groups were also prepared. For one group, only healthy

152   pearl oysters (N=7) were reared in an aquarium. For the other, supernatant of the soft body (4.86 g) from a single

153   healthy animal was prepared as described above and injected into the adductor muscles of 7 healthy oysters (100

154   μl/individual) without filtration. Experiments were performed in duplicate in all test groups including positive and

155   negative controls. The mortality was monitored, and the shell scores were determined on surviving individuals 38

156   days after the start of the experiment.

157

158   **Test 4: Serial passages**

159   To further clarify whether the disease was transmissible, we performed an experiment to know whether the

160   disease could be passed on repeatedly. The serial passages were conducted with 3 different intervals: 1h, 5days, and

161   10 days. The outline of the test is shown in Fig 4. Soft bodies collected from 10 0-year-old oysters of an affected

162   group were pooled (wet weight 4.0 g) and cut into small pieces with scissors. The volume of the minced tissues was

163   adjusted to 6 mL with autoclaved seawater and centrifuged at 1200 ×g for 10 min. The supernatant was filtered

164   through a 0.1 um filter (Millipore). The filtrate was injected into the adductor muscles of 25 healthy oysters (100

165   μl/individual); this infection test was defined as the first passage. From the 25 oysters, 5 individuals were randomly

166   selected at 1 hour, 5 days, and 10 days after the inoculation, and the hemolymph was collected and pooled to prepare

167   the inoculum for the second passage. The pooled hemolymph was centrifuged at 1200 ×g for 10 min to obtain the

168   supernatant as the second inoculum. The inoculum was injected into the adductor muscles of 12 healthy pearl

169   oysters (100 μl/individual) in each test series (total N=36). As for the third passage, 5 individuals were selected

170   from each group at 1 hour, 5 days, and 10 days after the second inoculation, and the hemolymph was collected and

171   used as the inoculum. This procedure was repeated up to the 3rd passage for the 1-hour interval passages, to the 4th

172   passage for the 5-day interval passages, and to the 8th passage for the 10-day interval passages. The remaining

173   oysters in each passage (10 individuals for the first passage and 7 for the other passages) were kept for 3 weeks to

174   observe shell abnormality. For negative controls, 7 healthy oysters were similarly inoculated with supernatant of

175   hemolymph that was prepared from 5 healthy animals. Serial passages were not conducted for the negative controls.

176

177   **Test 5: Chloroform sensitivity**

178   This test was performed twice (Table 1). As the inoculum in the first test (Test 5-1), the supernatant of the

179   hemolymph used for the infection of the first passage in Test 4 was used. In the second test (Test 5-2), the

180   supernatant of the hemolymph used for the third passage in the 10-day interval passages in Test 4 was used. In both

181   tests, the inoculum was divided into two equal aliquots, an equivalent volume of chloroform was added to one

182   aliquot, and the mixture was shaken at 25°C at 300 rpm for 10 minutes and then centrifuged at 1200 ×g for 10

183   minutes to recover the aqueous layer as the chloroform-treated inoculum. The other was similarly shaken and

184   centrifuged without adding chloroform and used as the non-treated inoculum. For the negative control, supernatant

185   of the hemolymph (centrifuged at 1200 x g for 10 min) of a single healthy oyster was used as the inoculum. Each

186   inoculum was injected into the adductor muscles of 5 and 7 healthy pearl oysters in the Tests 5-1 and 5-2,

187   respectively (100 μl/individual). The mortality was monitored during the experiments. The shell scores were

188   determined on the surviving individuals 15 and 20 days after the injections in the two tests, respectively.

189

190   **Test 6: Thermal stability**

191   This test was performed twice (Table 1). In the first test (Test 6-1), the supernatant of the hemolymph used for

192   the 7th passage in the 10-day interval passages in Test 4 was used as the inoculum. In the second test (Test 6-2), the

193   inoculum was prepared from 0-year-old juveniles of an affected group (Table 1); soft bodies were collected from 10

194   juveniles, pooled (0.8 g in total wet weight), chopped with scissors in 5 mL of autoclaved seawater, and centrifuged

195   at 1200 x g for 10 min to obtain the supernatant as the inoculum. For each experiment, the inoculum was dispensed

196   into 1.5 mL tubes (1.0 mL/tube). The tubes were incubated for 30 min in heat blocks maintained statically at four

197   different temperatures: 40°C, 50°C, 60°C, or on ice. Subsequently, all tubes were rapidly cooled in an ice bath. For

198   the negative controls, supernatant was prepared from hemolymph (Test 6-1) or macerated tissue (Test 6-2) of

199   healthy animals and used without heat treatment. Each inoculum was injected into the adductor muscles of 5 healthy

200   pearl oysters (100 μl/individual). The mortality was monitored during the experiments. The shell scores were

201   determined on the surviving individuals 21 and 15 days after the injections in Tests 6-1 and 6-2, respectively.

202

203   **Histopathology**

204   Sampling for histopathology was conducted on days 5 and 10 after the inoculation of the first passage in the

205   Test 4. For the 5-day samples, tissues were taken from the oysters used as donors for the 2nd passage for 5-day

206   interval passages. For the 10-day samples, tissues were taken from the animals used as donors for the 2nd passage

207   for 10-day interval passages. At each sampling, the mantle, gill, kidney, heart, adductor muscle, foot, and midgut

208   (including the stomach and gonads) were dissected from 5 specimens after collecting the hemolymph. The tissues

209   were fixed in Davidson's solution (Bell and Lightner, 1988), dehydrated through an ethanol series and embedded in

210   paraffin. Two sets of 3 μm sections were prepared from each tissue, stained with hematoxylin and eosin or May-

211   Grunwald / Giemsa, and observed under a microscope.

212

213   **Statistics**

214   The mean shell scores were expressed as means ± standard error. The mortality and prevalence of nacre

215   abnormalities in survivors were analyzed statistically with Fisher's exact test, and the mean shell scores were tested

216   by Mann-Whitney U-test or the Steel-Dwass test. A value of $p < 0.05$ was considered to be statistically significant.

217

Results

Investigation of spontaneously occurring disease in farmed oysters

In the oysters obtained from pearl farmers in Mie and Ehime prefectures where summer atrophy occurred, nacre abnormalities were observed in 45.6〜81.6% of the oysters (Table 2). Cultured pearl oysters from Mie prefecture before the disease occurred, and wild pearl oysters from areas where pearl farming has never performed and the disease has not been recorded, did not show the nacre abnormalities that are seen in the disease.

Test 1: Cohabitation and injection challenge using adult pearl oysters as the infection source

There were no significant differences in mortality between test groups (Table 3). Appearances of nacre abnormalities of the experimental groups were significantly higher than those of the corresponding negative controls, except for the cohabitation experiment at the low temperature (Table 3). Shell scores of the injection-challenged groups were significantly higher than those of the negative controls.

Test 2: Injection challenge using juvenile pearl oysters as the infection source

No mortality was observed in the negative control group (Table 4). In the group injected with inoculum prepared from diseased pearl oysters, death of the experimental animals started on the 11th day after inoculation, and 7 out of 10 individuals died by the end of the experiment. Six of the seven dead specimens exhibited shell abnormalities, and the mean shell score obtained for the seven dead individuals was 1.7 ± 0.4. The abnormalities on the nacre were also confirmed in all three surviving individuals, and the mean shell score for the three was 3.3 ± 0.7. No abnormalities were observed in the shells of the surviving individuals in the negative control group.

Test 3: Filterability

The results are summarized in Table 5. Mortality was only found in the experimental and positive control groups. Nacre abnormalities were also mostly observed in the experimental and positive control animals, although slight abnormalities were observed in some control oysters. Thus, the disease was clearly reproduced only in the experimental and positive control groups including the experimental oysters injected with the hemolymph filtered through 0.1 μm filter.

Test 4: Serial passages

Neither mortality nor nacre abnormalities were observed in the negative control group (Table 6). In the 1-hour interval series, shell abnormalities were observed by the second passage, but no abnormalities were observed in the 3rd passage. On the other hand, in the 5-day and 10-day interval passages, the nacre abnormalities were observed up to the end of the experiments.

Test 5: Chloroform sensitivity

In both experiments, the nacre abnormalities appeared similarly in the two experimental groups irrespective of the treatment of chloroform, whereas no abnormalities were found in the nacre of the control oysters (Table 7).

255

256 **Test 6: Thermal stability**

257 No abnormalities in the nacreous layer were observed in the groups injected with the inoculums incubated at

258 50°C and 60°C in Test 6-1, and in the group injected with the inoculum treated with heat at 60°C in Test 6-2 (Table

259 8).

260

261 **Histopathology**

262 In the oysters sampled on day 10, increased numbers of migrating cells were observed in the connective

263 tissues in 4 out of 5 individuals, but no other significant histopathological changes were found in any of the

264 observed specimens.

265

266 ## Discussion

267 The results of this study suggest that summer atrophy of Akoya pearl oyster is an infectious disease. The nacre

268 abnormalities found in spontaneously affected oysters were reproduced in healthy pearl oysters that were reared with

269 diseased oysters in 3 of the 4 experiments (Tests 1 and 2). Nacre abnormalities were also confirmed in the surviving

270 oysters in all six infection tests with injections of the inoculum prepared from affected pearl oysters.

271 The mass mortality of juveniles and abnormalities in adult oysters in pearl culture farms are likely to be

272 caused by the same causative agent, considering the results of the present study, in which the disease was

273 reproduced either by affected adults or by affected 0-year-old juveniles as the donors. Nevertheless, infection tests

274 on juveniles should be conducted in future studies to see if mass mortality could be reproduced, since only adult

275 oysters were used as recipients in this study.

276 The results of this study also suggest that the etiology of this disease is proliferative. Both in the 5-day and 10-

277 day interval passages, the nacre abnormalities were confirmed up to the end of the experiments. Therefore, the

278 etiological agent of this disease probably proliferates within 5-10 days after inoculation to the amount that is enough

279 to cause the disease in the next passage. In the 1-hour interval passages, however, none of the individuals in the

280 third passage developed the abnormalities of nacre surface, although the nacre abnormalities were confirmed up to

281 the second passage. The causative agent probably could not sufficiently propagate in 1 hour, and the disease

282 appeared in the second passage was most likely caused by the causative agent that had been carried over from the

283 injection in the first passage.

284 The etiological agent in the host body appears to decrease quickly as the oyster recovers. In previous studies, we

285 could not reproduce the disease either by cohabitation or injection when pearl oysters with normal soft bodies were

286 used as donors, despite that we obtained those donors from the group that had experienced summer atrophy and

287 confirmed typical nacre abnormalities in those animals (Supplemental Tables 1 and 2). Therefore, to reproduce the

288 disease, it seems important to use diseased oysters with atrophied soft bodies or oysters from populations that are

289 currently experiencing mass mortality as the infection source.

290 Considering the filterability (Test 3), the sensitivity to chloroform (Test 5) and the thermal stability (Test 6) of

291 the pathogen, the causative agent for summer atrophy seems to be a non-enveloped virus with a minor axis of less

292   than 100 nm, which is inactivated 50 or 60 $^{\circ}$C for 30min. In addition, the optimal temperature for the propagation of

293   the pathogen might be around 25 $^{\circ}$C or more, since the disease was not clearly reproduced by cohabitation at 22-23

294    $^{\circ}$C in this study. This is supported by the fact that the spontaneous disease outbreaks occur during summer as stated

295   before. However, this requires further study.

296   We could not find any noticeable histopathological changes in the affected pearl oysters in the present study. In

297   fact, we have not observed any substantial histopathological changes that are common to spontaneously diseased

298   oysters presumably affected with summer atrophy so far. This seems peculiar for a disease with mass mortality

299   caused by an infectious agent. It is possible that we have overlooked some important histological changes, although

300   it would be clarified if the pathogen is definitively identified.

301   Several cases similar to summer atrophy have been reported in the genus *Pinctada*, but none are identical to the

302   disease. Birnavirus (Suzuki et al., 1998a) and "Akoya virus" (Miyazaki et al., 1999) were reported to infect *P. fucata*

303   in Japan, although the symptoms of summer atrophy were not observed with those virus infections. Moreover,

304   neither biochemical nor morphological characteristics have been reported for Akoya virus, and its existence itself is

305   uncertain. Akoya virus was reported to be the causative agent of Akoya oyster disease (AOD), although its

306   relationship with AOD has been refuted (Matsuyama et al., 2017). Virus infections have also been reported in *P.*

307   *maxima* (Pass et al., 1988; Norton e al., 1993). However, the characteristic viral inclusion bodies observed in these

308   studies were not found in this study, and hence, these viruses seem to be unrelated to summer atrophy of *P. fucada*.

309   Mass mortalities of unknown etiology has been reported in the members of the genus *Pinctada* (Wolf and

310   Sprague, 1978, Nasr, 1982, Comps et al., 2001, Jones et al., 2010). One of those, "syndrome 85" is similar to

311   summer atrophy in *P. fucada*, in that juvenile experience mass mortality and show symptoms that include deposition

312   of brown organic matter on the inner surface of the two valves in *P. margaritifera* (Comps et al., 2001). Unlike

313   summer atrophy, however, high mortality occurs irrespective of the age of the shells in syndrome 85, and the disease

314   is also characterized by granulomatous tissue formation (Comps et al., 2001). Another abnormal condition with

315   unknown etiology is oyster oedema disease (OOD). The disease caused mass mortality in *P. maxima* in Western

316   Australia in 2006 (Jones et al., 2010). Similar to summer atrophy, the mantle of OOD-affected oysters is contracted,

317   and histological responses are not observed (Jones et al., 2010). However, edema does not occur in summer atrophy.

318   Thus, summer atrophy of *P. fucada* seems to be a new infectious disease in the genus *Pinctada*, although it is

319   possible that the differences in the symptoms in those diseases with unknown etiology in *Pinctada* might be due to

320   the difference in the host species.

321   Abnormalities in shells of bivalves, which are often collectively called "shell disease", can be caused by a variety

322   of organisms, such as parasites (Martinelli et al., 2020, Farley, 1968), fungi (Friedman et al., 1997, Raghukumar and

323   Lande, 1988, Renault et al., 2002), bacteria (Elston and Leibovitz, 1980, Paillard and Maes, 1995, Elston et al., 1999,

324   Boettcher et al. 2005, Huang et al., 2019), and viruses (Momoyama et al., 1999, Matsuyama et al., 2020). Shell

325   disease in *P. fucata* has also been experimentally reproduced by inoculating viable *Escherichia coli* or the yeast

326   *Saccharomyces cerevisiae* into the extrapallial space of the valves (Huang et al., 2019). Since the responsible tissue

327   for shell formation is the mantle, these pathogens probably affect the activity of mantle, either directly or indirectly,

328   and lead to shell disease. Sano et al. (2021) compared the expression levels of five genes in the mantle of healthy *P.*

329   *fucata* and the animals affected with summer atrophy, and found decreased expression of the *OT47* gene (tyrosinase

330   gene involved in the formation of periostracum (Zhang et al., 2006)) and increased expression of the *msi31* gene

331   (shell matrix protein gene involved in the formation of the prismatic layer (Sudo et al., 1997)) in the diseased oysters.

332   The expression levels of these genes may be altered by the causative agent of the disease in the mantle. However, no

333   obvious histopathological change has been observed in the tissues of the diseased pearl oysters, including the mantle,

334   so far. It is therefore unknown whether the etiological agent of the disease exists in the mantle. To resolve these

335   issues and to identify the etiological agent, we are currently conducting experiments utilizing next-generation

336   sequencing.

337

Conclusions

339   This study was conducted to clarify the involvement of infectious agents in the mass mortality in juveniles and

340   atrophy and mortality in adult pearl oysters (*P. fucata*) in the major pearl farming areas in Japan in 2019 and 2020.

341   The condition, or the disease, is referred to as "summer atrophy" here. The oysters recovered from the disease

342   exhibited deposition of brown materials and loss of luster on the inner nacreous surface of valves, which is usually

343   smooth and shiny. These shell abnormalities were reproduced in healthy pearl oysters either by injecting

344   hemolymph from affected oysters or by cohabitation with diseased specimens. The disease was successfully

345   reproduced through continual passages using hemolymph as the inoculum up to the 8th passage. The cause of this

346   disease is hence considered to be a proliferative pathogen. In addition, pathogenicity was not affected by filtering the

347   inoculum through a 0.1 μm filter or by chloroform treatment. Therefore, the etiological agent is likely to be a non-

348   enveloped virus with a diameter of less than 100 nm. Definitive identification of this pathogen is the ongoing subject

349   of our study.

350

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354   for their assistance for sampling pearl oyster.

355

Figure legends

357

358   Figure 1. Locations of major pearl farms and areas where healthy pearl oysters were obtained.

359   The prefectures painted in red and black indicate major pearl farming areas using *P. fucata* and areas where

360   healthy pearl oysters were obtained, respectively.

361

362   Figure 2. Appearances of diseased and healthy pearl oysters.

363   (A) A 1-year-old pearl oyster with summer atrophy. The soft body is atrophied and is contracted towards the

364   dorsal margin. The arrowhead indicates brown deposit on the nacre along the mantle edge. (B) Nacre of 2-year-old

365   pearl oysters affected by summer atrophy. Arrowheads indicate brown deposit on the nacre. (C) Zero-year-old pearl
366   oysters affected by summer atrophy. Deposition of brownish material is seen on the nacre surface (white
367   arrowheads), whereas shell spines are extending (yellow arrowheads). (D) A normal pearl oyster. The mantle covers
368   the entire nacre surface. (E) A pearl oyster with black-spot shell disease. Dark, organic matter is deposited mainly
369   from the dorsal edge to the central part on the nacre. This individual was sampled from the group affected by
370   summer atrophy. Loss of luster (the white arrowhead) and slight pigmentation (the yellow arrowhead) seem to be
371   the symptoms of summer atrophy. (F) A Pearl oyster with Akoya oyster disease. The soft body is atrophied and has
372   turned reddish-brown in color. No abnormality is observed on the nacre.

373

374   Figure 3. Scoring the severity of disease
375   The severity of the disease was quantified according to the ratio of abnormal areas (deposition of brown pigment
376   and loss of luster) to the total nacreous surface: score 0, normal; score 1, the abnormal area is less than 30%; score 2,
377   the abnormal area is larger than 30% and less than 70%; and score 3, the abnormal area is larger than 70%.
378   In these photographs, the shell with score 1 has brown deposit (the white arrowheads) on the upper right, and
379   the shell with score 2 has brown deposit on the upper left and bottom. In the shell with score 3, deposition was
380   observed on the upper and lower areas where the mantle was located, and the surrounding large area of nacre has
381   lost its luster (the yellow arrowheads).

382

383   Figure 4. Outline of the transmission experiments with serial passages
384   The serial passages were conducted with 3 different intervals: 1 hour, 5 days, and 10 days. Hemolymph was
385   collected from 5 specimens in each cohort, pooled, and injected into the next group. The
386   remaining 7 individuals in each cohort were sampled 3 weeks after inoculation to observe
387   abnormalities of nacre surface.

388

Supplemental data

389
390
391   Infection tests using pearl oysters with shell   abnormalities and normal soft bodies as the infection source.

392

393

394   Materials and Methods

395

396   1. Animals
397   One-year-old, healthy pearl oysters from Ishikawa Prefecture were used as the recipients. Two groups of
398   oysters of different ages (1 and 2-year-old) that had experienced summer atrophy were obtained from 2 pearl oyster
399   farms respectively in Mie Prefecture and used as the donors. The experiments were performed in 65L tanks filled
400   with 56 L of running seawater maintained at 25°C and filtered with a 1-μm pore size filter. The oysters were fed
401   with diatoms (*Chaetoceros neogracile*), five times a week.

402

403 2. Infection test by cohabitation

404   Before the start of the test, five oysters from each of the donor groups and from the recipient group were

405 randomly sampled and the shell scores were determined. Cohabitation tests were conducted separately, either by

406 using 1-year-old oysters or 2-year-old oysters as the donors. Experimental tanks were duplicated for each donor

407 group. The number of donor oysters in each tank was 4 for 1-year-old and 10 for 2-year-old animals. Twenty healthy

408 oysters were reared with donors in each tank. For the negative control, ten healthy oysters were used as donors and

409 reared similarly with 20 healthy oysters in the same tank. The negative control tanks were also duplicated. Five

410 recipient individuals from each tank were sampled to determine the shell scores on days 42, 63, and 111 after the

411 start of cohabitation, and the mean shell scores were calculated.

412

413 3. Infection test by hemolymph injection

414   Hemolymph was collected from the adductor muscles of 10 affected oysters of the 2-year-old group. The shell

415 scores for these oysters were determined after collecting the hemolymph. The hemolymph was pooled, and the

416 supernatant was obtained by centrifugation at 1200 $\times g$ for 10 min. The supernatant was injected into the adductor

417 muscles of 20 healthy oysters (100 μL/oyster). For the negative control, the same volume of autoclaved seawater

418 was injected into the adductor muscles of 20 healthy oysters and the oysters were similarly reared thereafter. Both

419 experimental and control tanks were duplicated. On the 51st day after the injections, 10 individuals from each group

420 were sampled and the mean shell score was calculated.

421

422 Results

423   1. Infection test by cohabitation

424 The donor oysters showed no atrophy in the soft body. Nevertheless, abnormalities were observed on the nacre of all

425 specimens. The mean shell scores for the 1- and 2-year-old, affected pearl oysters were 4.2 ± 0.7 and 3.2 ± 0.9,

426 respectively. These donor animals appeared to have recovered from atrophy. The mean shell scores for the recipient

427 oysters after cohabitations with the donors was 0 ± 0 to 1.0 ± 0.5, which were not significantly different from those

428 for the negative control groups (Supplemental Table 1).

429

430   2. Infection test by hemolymph injection

431   While none of the pearl oysters used for hemolymph collection exhibited atrophy of the soft body, the nacre of

432 all oysters was abnormal. The mean shell score for the donor oysters was 5.0 ± 0.6 (N=10). The mean shell scores

433 for the two recipient groups at the end of the experiment, however, were 0 ± 0 and 0.2 ± 0.1, which were not

434 significantly different from the value of 0 ± 0 for the negative control groups (Supplemental Table 2).

435

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514

Figure 1

Locations of major pearl farms and areas where healthy pearl oysters were obtained.

The prefectures painted in red and black indicate major pearl farming areas using *P. fucata* and areas where healthy pearl oysters were obtained, respectively.

Ishikawa

Kanagawa

Nagasaki

Ehime

Mie

Wakayama

Ōita

Kumamoto

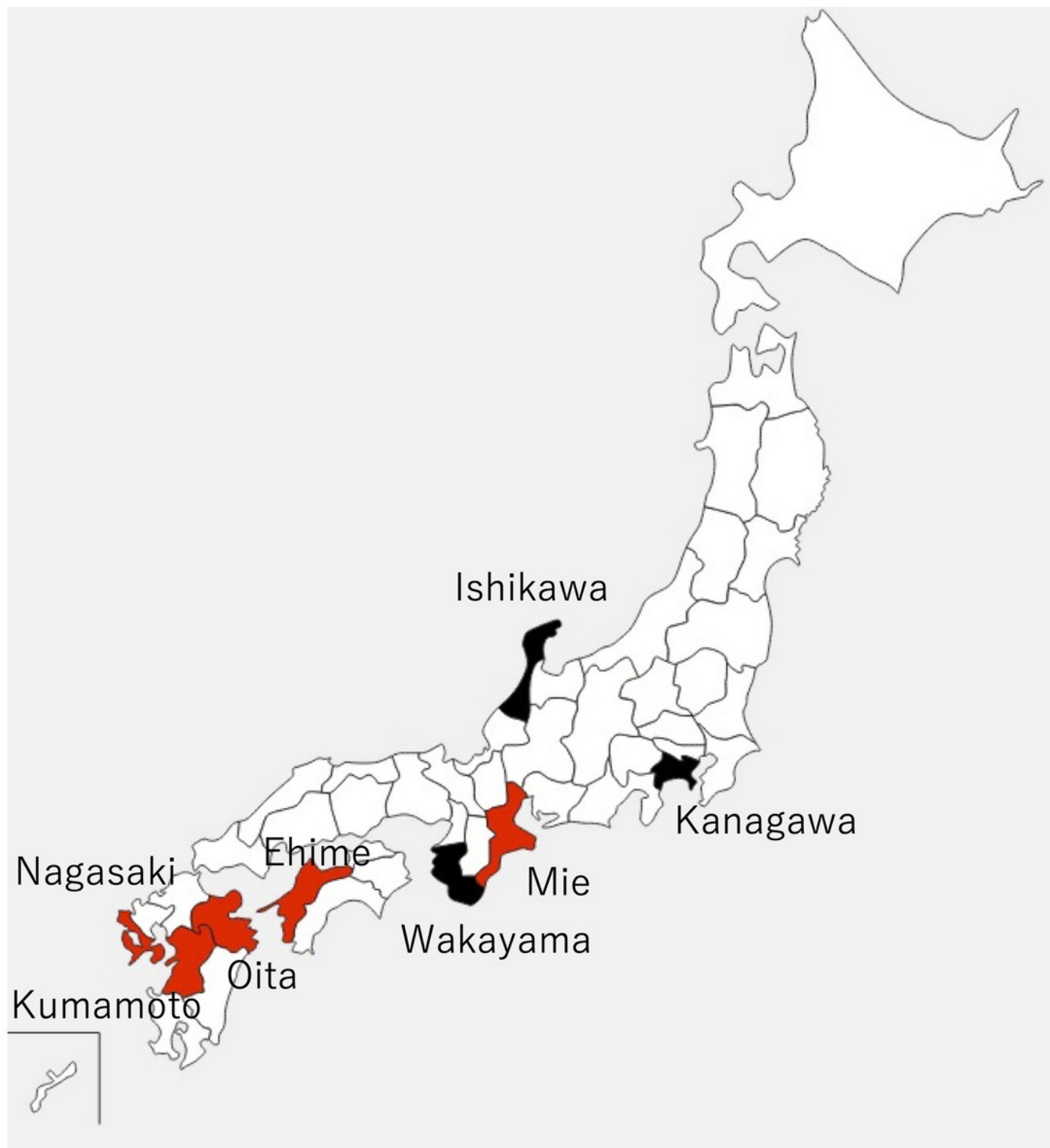



Figure 2

Appearances of diseased and healthy pearl oysters.

(A) A 1-year-old pearl oyster with summer atrophy. The soft body is atrophied and is contracted towards the dorsal margin. The arrowhead indicates brown deposit on the nacre along the mantle edge. (B) Nacre of 2-year-old pearl oysters affected by summer atrophy. Arrowheads indicate brown deposit on the nacre. (C) Zero-year-old pearl oysters affected by summer atrophy. Deposition of brownish material is seen on the nacre surface (white arrowheads), whereas shell spines are extending (yellow arrowheads). (D) A normal pearl oyster. The mantle covers the entire nacre surface. (E) A pearl oyster with black-spot shell disease. Dark, organic matter is deposited mainly from the dorsal edge to the central part on the nacre. This individual was sampled from the group affected by summer atrophy. Loss of luster (the white arrowhead) and slight pigmentation (the yellow arrowhead) seem to be the symptoms of summer atrophy. (F) A Pearl oyster with Akoya oyster disease. The soft body is atrophied and has turned reddish-brown in color. No abnormality is observed on the nacre.

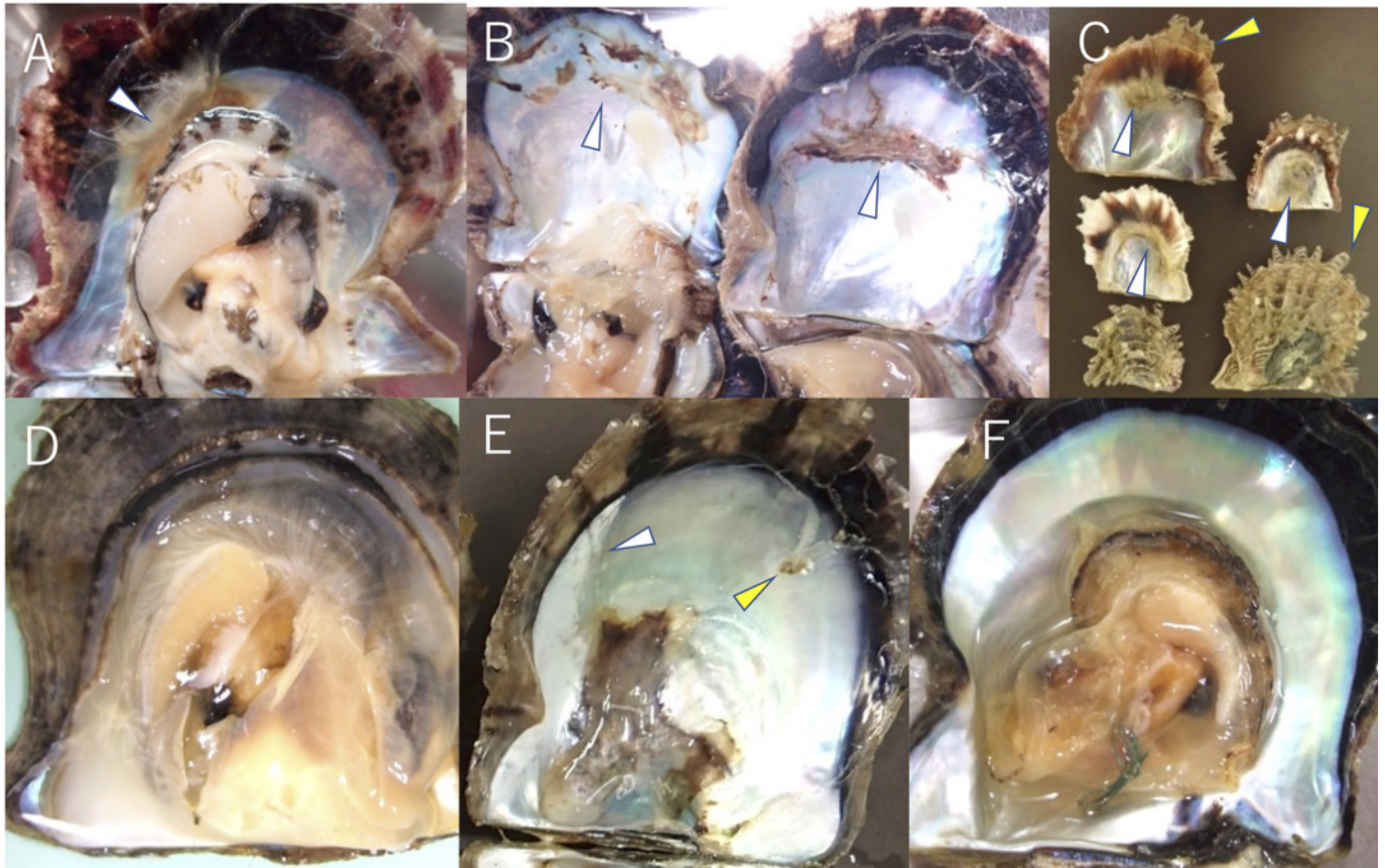
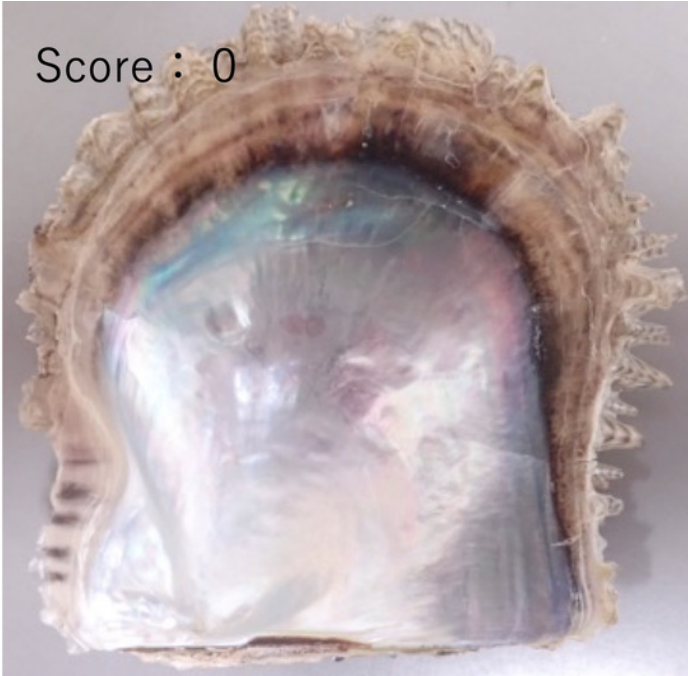



Figure 3

Scoring the severity of disease

The severity of the disease was quantified according to the ratio of abnormal areas (deposition of brown pigment and loss of luster) to the total nacreous surface: score 0, normal; score 1, the abnormal area is less than 30%; score 2, the abnormal area is larger than 30% and less than 70%; and score 3, the abnormal area is larger than 70%. In these photographs, the shell with score 1 has brown deposit (the white arrowheads) on the upper right, and the shell with score 2 has brown deposit on the upper left and bottom. In the shell with score 3, deposition was observed on the upper and lower areas where the mantle was located, and the surrounding large area of nacre has lost its luster (the yellow arrowheads).

Score:0

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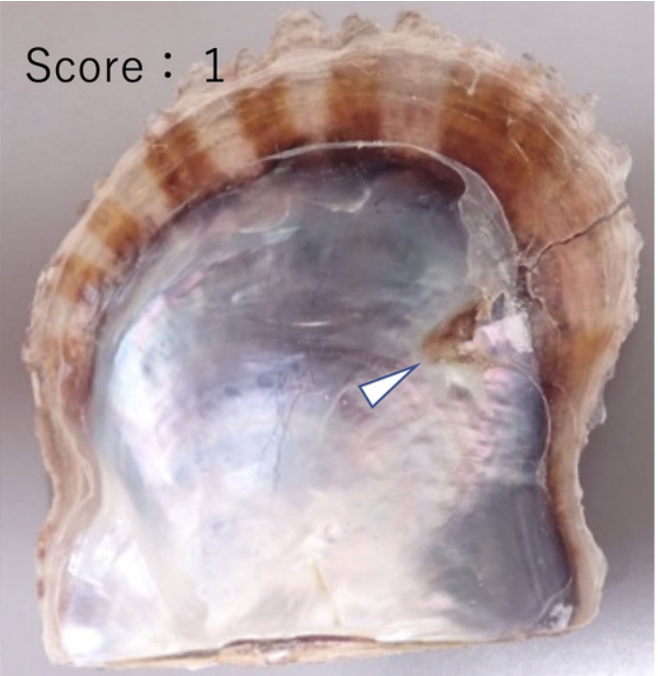
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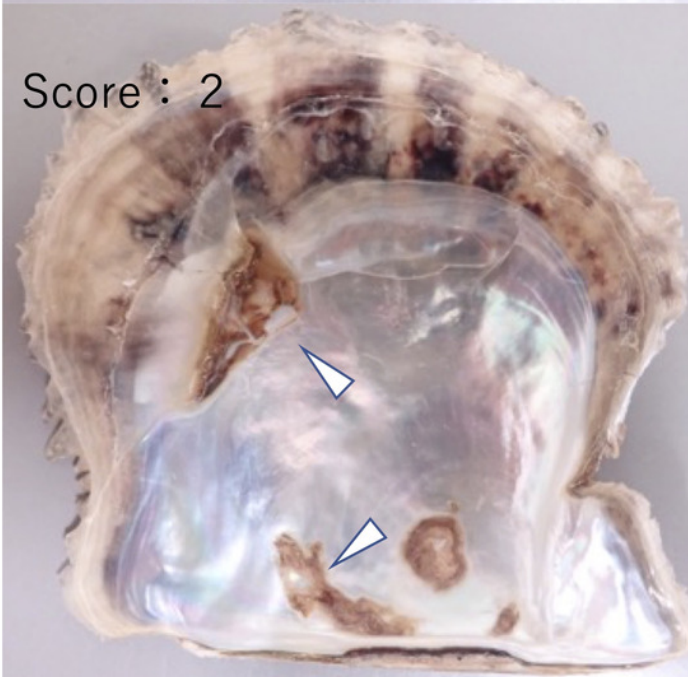
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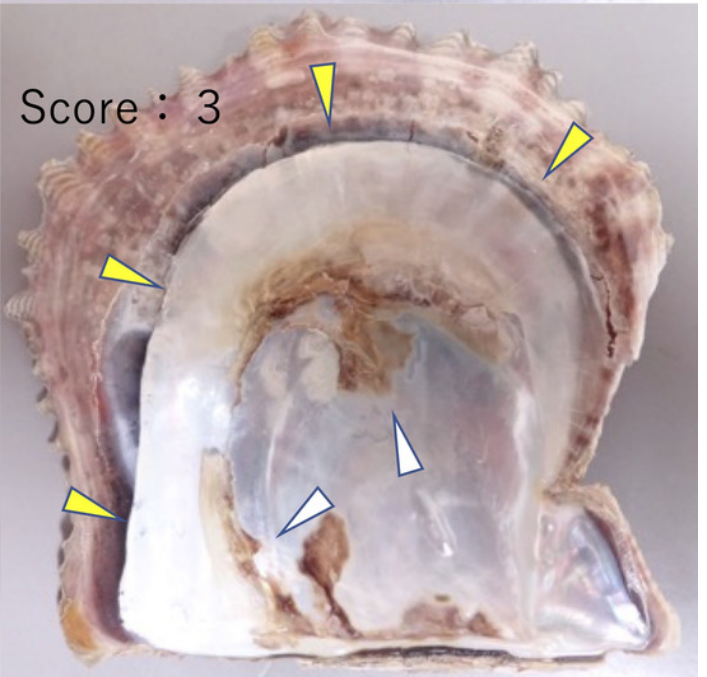
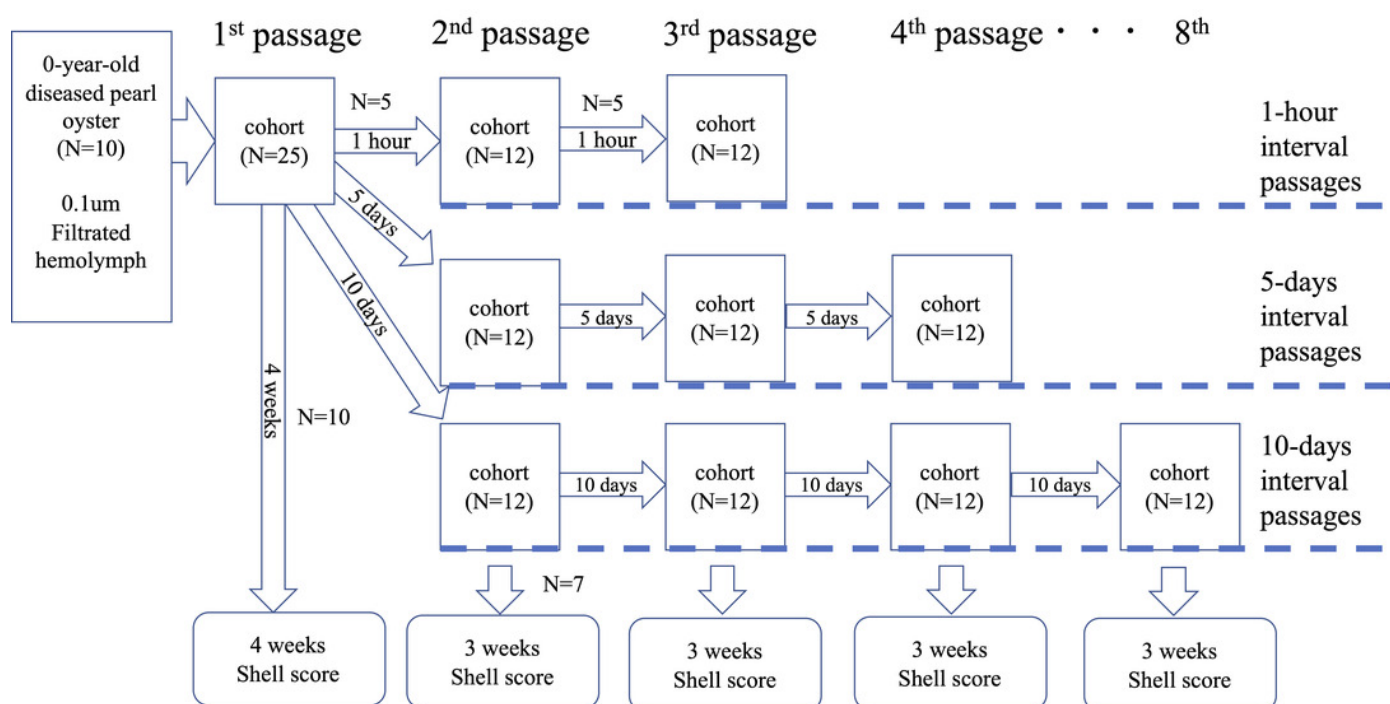


Figure 4

Outline of the transmission experiments with serial passages

The serial passages were conducted with 3 different intervals: 1 hour, 5 days, and 10 days. Hemolymph was collected from 5 specimens in each cohort, pooled, and injected into the next group. The remaining 7 individuals in each cohort were sampled 3 weeks after inoculation to observe abnormalities of nacre surface.

Summary of pearl oysters used

*: The number of oysters in which nacre abnormality was observed / the number of oysters sacrificed from each group immediately before the start of the experimets. No atrophy in the soft tissues was observed in the recipient groups. #: The number of oysters in which the soft body was atrophied / the number of oysters sacrificed from each group immediately before the start of the experiments. Nacre abnormalities remain after the oyster recovered from the disease, and hence, it cannot be used to judge if the oyster is diseased at the time of observation. NM: Not measured

Table 1. Summary of pearl oysters used

Healthy pearl oysters (Recipients)

Infection xperiments	Collection sites	Age (years old)	Range in shell width (mm, mean ± SD)	Prevalence of atrophy[#]	Nacre abnormalities [*]	Mean shell score
Test 1	Ishikawa	1	45 - 57 (49.3 ± 3.7)	0/10	0/10	0 ± 0
Test 2	Ishikawa	1	51 - 63 (59.6 ± 3.1)	0/10	2/10	0.4 ± 0.3
Test 3	Kanagawa	1	45 - 56 (49.8 ± 3.6)	0/5	0/5	0 ± 0
Test 4	Kanagawa	1	55 - 68 (61.8 ± 2.3)	0/10	0/10	0 ± 0
Test 5-1	Kanagawa	1	ditto	0/10	0/10	0 ± 0
Test 5-2	Kanagawa	1	51 - 64 (55.0 ± 5.6)	0/5	0/5	0 ± 0
Test 6-1	Kanagawa	2	51 - 65 (57.6 ± 1.4)	0/5	0/5	0 ± 0
Test 6-2	Kanagawa	3	50 - 75 (15.8 ± 0.8)	0/5	1/20	0.1 ± 0.1

Source of infection (Donors)

Infection experiments	Collection sites	Age (years old)	Range in shell width (mm, mean ± SD)	Prevalence of atrophy[#]	Nacre abnormalities [*]	Mean shell score	Source of inoculum	Note

Test	Group		Size range (mean)	*	#		Inoculum	Remarks
Test 1	Mie	3	73 - 60 (65.0 ± 1.5)	3/10	7/10	2.3 ± 0.6	Hemolymph	There are traces of previous year.
Test 2	Mie	0	NM	5/10	0/10	0 ± 0	Supernatant of macerated tissue	Cumulative mortality rate at the time of sampling was ca. 20%. No deaths up to 6 days before sampling.
Test 3	Mie	0	27 - 32 (29.2 ± 1.2)	2/10	4/10	1.3 ± 1.1	Supernatant of macerated tissue	Nine days before the experiment, 9 out of 10 individuals were atrophied. Mortality rate unknown.
Test 4	Mie	0	26 - 45 (33.5 ± 2.0)	2/10	NM	NM	Supernatant of macerated tissue	The same lot as in Test 3 were used.
Test 5-1	Mie	0	28 - 41 (32.0 ± 3.3)	2/10	NM	NM	Supernatant of macerated tissue	The same lot as in Test 3 were used.
Test 5-2	Experimentally infected	1	50 - 67 (58.6 ± 3.1)	4/5	0/5	0 ± 0	Hemolymph	Obtained from Test 4
Test 6-1	Experimentally infected	1	52 - 61 (56.0 ± 2.6)	1/3	0/3	0 ± 0	Hemolymph	Obtained from Test 4
Test 6-2	Mie	0	12 - 20 (15.8 ± 0.8)	4/10	7/10	1.7 ± 0.5	Supernatant of macerated tissue	Cumulative mortality rate at the time of sampling was ca. 33.3%. No deaths up to 2 days before sampling.

*: The number of oysters in which nacre abnormality was observed / the number of oysters sacrificed from each group immediately before the start of the experimets. No atrophy in the soft tissues was observed in the recipient groups.

#: The number of oysters in which the soft body was atrophied / the number of oysters sacrificed from each group immediately before the start of the experiments. Nacre abnormalities remain after the oyster recovered from the disease, and hence, it cannot be used to judge if the oyster is diseased at the time of observation.

NM: Not measured

Prevalence of atrophy and nacre abnormalities of the pearl oysters collected in different regions and ages

Nacre abnormalities and mean shell score were calculated for the incidence and score per shell, not per individual. □: The number of oysters in which the soft body was atrophied / the number of oysters sacrificed from each group immediately before the start of the experiments. Nacre abnormalities remain after the oyster recovered from the disease, and hence, it cannot be used to judge if the oyster is diseased at the time of observation. □: The number of valves in which nacre abnormality was observed / the number of tested valves from each group. !: Valves stored in a dry state at room temperature were observed on 2021.7.6. †: Nature of pigmentation was much darker than those typically found in the oysters affected by summer atrophy and rather similar to that of black-spot shell disease. NM: Not measured

Table 2 Prevalence of atrophy and nacre abnormalities of the pearl oysters collected in different regions and ages □

Collection dates	Collection sites	Age (years old)	Range in shell width (mean ± SD)	Prevalence of atrophy *	Nacre abnormalities/valve #	Mean shell score/valve	Note
Summer atrophy-free area							
2019.8.23	Ishikawa	1	49 - 67 (58.5 ± 1.0)	0/40	0/80 (0%)	0 ± 0	
2020.6.4	Ishikawa	1	42 - 66 (56.0 ± 1.8)	0/40	2/40 (5%)	0.05 ± 0.04	
2019.10.22	Wakayama	0-2	29 - 55 (44.6 ± 2.5)	0/16	0/32	0 ± 0	
2019.11.1	Wakayama	0-2	29 - 55 (44.6 ± 2.5)	0/20	0/40	0 ± 0	
2020.9.10	Kanagawa	1	45 - 60 (58.8 ± 5.8)	0/30	1/60[†] (1.7%)	0.03 ± 0.02	
2020.10.21	Kanagawa	1	51 - 73 (61.9 ± 3.8)	0/30	0/60	0 ± 0	
Summer atrophy-epidemic area							
Before summer atrophy epidemic							
2014.4.15 [!]	Mie	2	53-71 (59.8 ± 1.2)	NM	0/19	0 ± 0	
2014.7.19 [!]	Mie	2	53-64 (53.3±2.3)	NM	0/42	0 ± 0	
2017.4.21 [!]	Mie	1	39-54 (46.2±0.6)	NM	1/82[†] (2.4%)	0.05 ± 0.05	
2017.5.1 [!]	Mie	1	60-74 (64.2 ±1.1)	NM	1/17[†] (0.6%)	0.1 ± 0.1	
2019.4.25[!]	Mie	1					
After summer atrophy epidemic							
2019.8.23	Mie	1	41 - 55 (48.5 ± 1.7)	5/10	16/20 (80%)	1.9 ± 0.4	The cumulative mortality rate of 0-year-old oysters at the end of the mass mortality was more than 50%.
2019.9.19	Mie	0	27- 42	NM	17/30	1.2 ± 0.3	Randomly sampled, independent of

Date	Location	Score	Shell height (mm)	*	#	Mean shell score	Remarks
			(34.8 ± 0.7)		(56.7%)		disease onset.
2019.10.24	Mie	0 and 1	14 - 58 (29.9 ± 3.0)	NM	31/38 (81.6%)	1.6 ± 0.2	The cumulative mortality rate of 0-year-old oysters at the end of the mass mortality was ca.70%.
2019.11.20	Mie	1	50 - 57 (53.6 ± 0.5)	NM	11/20 (55%)	1.3 ± 0.3	Randomly sampled, independent of disease onset.
2020.3.11	Mie	1	50 - 68 (56.1 ± 1.4)	0/10	13/20 (65%)	1.1 ± 0.3	Randomly sampled, independent of disease onset.
2020.6.24	Mie	1	53 - 69 (59.0 ± 0.6)	7/44	24/44 (54.5%)	0.9 ± 0.2	Obtained a few days after the pearl farmer found the atrophy. Cumulative mortality rate at the time of sampling was ca. 20%.
2021.6.23	Mie	1	25 - 66 (47.8 ± 1.8)	NM	22/36 (61.1%)	1.1 ± 0.2	Obtained a few days after the pearl farmer found the atrophy.
2019.8.29	Ehime	1	24 - 58 (39.5 ± 2.3)	NM	33/48 (68.8%)	1.3 ±0.2	The cumulative mortality rate of 0-year-old oysters reared at the farm at the time of sampling was ca. 33.3%.
2019.10.17!	Ehime	0	26 - 45 (39.4 ± 0.4)	NM	39/68 (45.6%)	1.2 ± 0.2	Randomly sampled, independent of disease onset.
2019.10.17!	Ehime	1	50 - 70 (59.9 ± 0.6)	5/30	36/60 (60%)	1.4 ± 0.2	Randomly sampled, independent of disease onset.
2020.9.17!	Ehime	1-2	51 - 75 (61.3 ± 2.8)	NM	7/10 (70%)	1.4 ± 0.4	Randomly sampled, independent of disease onset.

Nacre abnormalities and mean shell score were calculated for the incidence and score per shell, not per individual.

*: The number of oysters in which the soft body was atrophied / the number of oysters sacrificed from each group immediately before the start of the experiments. Nacre abnormalities remain after the oyster recovered from the disease, and hence, it cannot be used to judge if the oyster is diseased at the time of observation.

#: The number of valves in which nacre abnormality was observed / the number of tested valves from each group.

!: Valves stored in a dry state at room temperature were observed on 2021.7.6.

†: Nature of pigmentation was much darker than those typically found in the oysters affected by summer atrophy and rather similar to that of black-spot shell disease.

NM: Not measured

Test 1: Cohabitation or injection with infected adult pearl oysters as the source of infection at two water temperatures

*: Significantly different from the negative control group (Fisher's exact test p < 0.05) #:

Significantly different from the negative control group (Mann-Whitney U-test p < 0.05) ᴵ; The

number of oysters in which nacre abnormality was observed/the number of surviving oysters

Table 3 Test 1: Cohabitation or injection with infected adult pearl oysters as the source of infection at two water temperatures

Water temperature 22 - 23°C

	Infection experiments			Negative controls	
	Cohabitation	Injection		Cohabitation	Injection
Mortality (dead/examined)	1/14	2/14		2/14	1/14
Nacre abnormalities[!]	2/13	9/12 *		1/12	1/13
Mean shell scores of survivors	0.5 ± 0.3	2.8 ± 0.4[#]		0.3 ± 0.1	0.07 ± 0.07

Water temperature 25°C

	Infection experiments			Negative controls	
	Cohabitation	Injection		Cohabitation	Injection
Mortality (dead/examined)	0/14	1/14		1/14	0/14
Nacre abnormalities[!]	5/14 *	9/13 *		1/13	0/14
Mean shell scores of survivors	0.9 ± 0.4	2.3 ± 0.4[#]		0.07 ± 0.07	0 ± 0

*: Significantly different from the negative control group (Fisher's exact test p < 0.05)

#: Significantly different from the negative control group (Mann-Whitney U-test p < 0.05)

[!]; The number of oysters in which nacre abnormality was observed/the number of surviving oysters

Test 2: Injection with juvenile pearl oysters as the source of infection

*: Significantly different from the negative control group (Fisher's exact test p < 0.05) [1]; The number of oysters in which nacre abnormality was observed/the number of observed oysters

Table 4   Test 2: Injection with juvenile pearl oysters as the source of infection

	Infection experiments	Negative controls
Mortality (dead/examined)	7/10*	0/10
Nacre abnormalities of dead [!]	6/7	-
Nacre abnormalities of survivor [!]	3/3	0/10
Mean shell scores of dead oysters	1.7 ± 0.4	-
Mean shell scores of survivors	3.3 ± 0.7	0 ± 0

*: Significantly different from the negative control group (Fisher's exact test p < 0.05)

[!]; The number of oysters in which nacre abnormality was observed/the number of observed oysters

Test 3: Filterability of causative agent of summer atrophy of pearl oyster

*: Significantly different from healthy oyster hemolymph - injected group (Fisher's exact test, p < 0.05). #: Significantly different from healthy oyster hemolymph - injected group in the same experimental series (Steel - Dwass test, p < 0.05). ¹; The number of oysters in which nacre abnormality was observed/the number of surviving oysters

Table 5. Test 3: Filterability of causative agent of summer atrophy of pearl oyster

	Pore size of membrane filters (μm) used for filtration of the hemolymph of affected oysters					Negative controls			Positive control
						Cohabitation with healthy oysters	Injection with healthy oyster hemolymph		Cohabitation with affected oysters
	0.8	0.45	0.22	0.1	□			□	
Experimental series-1									
Total number of deaths	1	0	1	1		0	0		1
Nacre abnormalities[!]	6/6*	7/7*	6/6*	5/6*		0/7	0/7		5/6*
Mean shell scores of survivors	3.3 ± 0.7#	3.9 ± 0.8#	4.2 ± 0.5#	3.0 ± 0.8#		0 ± 0	0 ± 0		2.8 ± 0.9#
Experimental series-2									
Total number of deaths	2	1	3	1		0	0		2
Nacre abnormalities[!]	5/5*	6/6*	3/4*	6/6*		2/7	0/7		5/5*
Mean shell scores of survivors	3.4 ± 0.7#	2.5 ± 0.5#	3.3 ± 1.1#	4.3 ± 0.2#	□	0.3 ± 0.2	0 ± 0	□	3.2 ± 0.5#

*: Significantly different from healthy oyster hemolymph - injected group (Fisher's exact test, $p < 0.05$).

#: Significantly different from healthy oyster hemolymph - injected group in the same experimental series (Steel - Dwass test, $p < 0.05$).

[!]; The number of oysters in which nacre abnormality was observed/the number of surviving oysters

Test 4: Mortality and disease appearances in serial passages

Blanks indicate that the experiments were not conducted. NM: not measured Passage 1 is common to all of the passage test series performed (i.e., every hour, 5 days, and 10 days) *: Significantly different from a negative control (Fisher's exact test, $p < 0.05$) #: Significantly different from a negative control (Steel-Dwass test, $p < 0.05$) ¦; The number of oysters in which nacre abnormality was observed/the number of surviving oysters

Table 6.   Test 4: Mortality and disease appearances in serial passages

	Negative control				1-hour passage series		
No. of passages	Dead/examined	Nacre abnormalities[!]	Mean shell scores of survivors	☐ Dead/examined	Nacre abnormalities[!]	Mean shell scores of survivors
1	0/7	0/7	0 ± 0	0/10	10/10*	$3.9 \pm 0.6^{\#}$
2				0/7	6/7*	3.0 ± 0.6
3	☐	☐	☐	0/7	0/7	0 ± 0

	5-days passage series				10-days passage series		
No. of passages	Dead/examined	Nacre abnormalities[!]	Mean shell scores of survivors	☐ Dead/examined	Nacre abnormalities[!]	Mean shell scores of survivors
1	0/10	10/10*	$3.9 \pm 0.6^{\#}$	0/10	10/10*	$3.9 \pm 0.6^{\#}$
2	0/7	7/7*	$4.4 \pm 0.5^{\#}$	0/7	7/7*	$5.0 \pm 0.5^{\#}$
3	0/7	6/7*	$3.1 \pm 0.8^{\#}$	3/7	3/4*	1.8 ± 0.6
4	0/7	6/7*	$3.3 \pm 0.7^{\#}$	0/7	6/7*	1.9 ± 0.6
5				0/7	6/7*	$3.5 \pm 0.8^{\#}$
6				NM	NM	NM
7				NM	NM	NM
8	☐	☐	☐	1/7	7/7*	$4.0 \pm 0.9^{\#}$

Blanks indicate that the experiments were not conducted. NM: not measured

Passage 1 is common to all of the passage test series performed (i.e., every hour, 5 days, and 10 days)

*: Significantly different from a negative control (Fisher's exact test, $p < 0.05$)

#: Significantly different from a negative control (Steel-Dwass test, $p < 0.05$)

[!]; The number of oysters in which nacre abnormality was observed/the number of surviving oysters

Test 5: Sensitivity to chloroform of causative agent

*: Significantly different from the group injected with healthy pearl oyster hemolymph (Steel -

Dwass test, p < 0.05) ¹; The number of oysters in which nacre abnormality was observed/the

number of surviving oysters

Table 7. Test 5: Sensitivity to chloroform of causative agent

Inoculum	Test 5-1		Test 5-2	
	Nacre abnormalities[!]	Mean shell score of survivors	Nacre abnormalities[!]	Mean shell score of survivors
Chloroform-treated	5/5	$2.8 \pm 0.9^*$	3/7	1.0 ± 0.6
Non-treated	5/5	$3.6 \pm 0.8^*$	3/7	1.4 ± 0.8
Hemolymph from healthy pearl oyster	0/5	0 ± 0	0/5	0 ± 0

*: Significantly different from the group injected with healthy pearl oyster hemolymph (Steel - Dwass test, $p < 0.05$)

[!]; The number of oysters in which nacre abnormality was observed/the number of surviving oysters

Test 6: Thermal stability of causative agent of suer atrophy of pearl oyster pathogenicity

*: Significantly different from healthy oyster hemolymph - injected group (Fisher's exact test, p < 0.05). #: Significantly different from healthy oyster hemolymph - injected group (Steel - Dwass test, p < 0.05). ¦; The number of oysters in which nacre abnormality was observed/the number of surviving oysters

Table 8. Test 6: Thermal stability of causative agent of summer atrophy of pearl oyster pathogenicity

	Temperature				Negative control
	on-ice	40°C	50°C	60°C	Injection with healthy oyster hemolymph
Test-1					
Nacre abnormalities[!]	5/5*	4/5*	0/5	0/5	0/5
Mean shell scores of survivors	$2.0 \pm 0.4^{\#}$	2.0 ± 0.6	0 ± 0	0 ± 0	0 ± 0
Test-2					
Nacre abnormalities[!]	4/5*	3/5*	4/5*	0/5	0/5
Mean shell scores of survivors	2.8 ± 0.9	2.2 ± 0.8	2.4 ± 0.8	0 ± 0	0 ± 0

*: Significantly different from healthy oyster hemolymph - injected group (Fisher's exact test, p < 0.05).

#: Significantly different from healthy oyster hemolymph - injected group (Steel - Dwass test, p < 0.05).

!; The number of oysters in which nacre abnormality was observed/the number of surviving oysters