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1 **Histological characteristics of the oocyte chorion in wild post-spawning and artificially matured**

2 **Japanese eels *Anguilla japonica***

3

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45

46 **Abstract**

47 To describe the histological characteristics of the oocyte chorion in wild adult and artificially
48 matured Japanese eels, we investigated changes in chorion thickness during artificially induced
49 oogenesis and compared the chorion thickness and ultrastructure between wild and artificially. In
50 artificially maturing eels, the chorion thickness and volume increased significantly with increasing
51 follicle diameter, peaking at approximately 450 μm ; beyond this point, the chorion thinned
52 significantly, whereas there were no significant changes in volume. A significant positive correlation
53 was observed between the number of salmon-pituitary-extract (SPE) injections and chorion thickness.
54 In wild post-spawning adult eels, chorion thickness varied among individuals, and two had chorions
55 that were significantly thinner than those of artificially matured eels. Ultrastructural examination
56 revealed electron-dense layers were observed in the chorions of wild post-spawning adult eels as was
57 seen in artificially matured eels. This result is inconsistent with our hypothesis that the formation of
58 an electron-dense layer is unique to artificially maturing eels due to repeated SPE injections. These
59 results suggest that the formation cycle of the chorion might be affected by SPE injections in artificially
60 maturing eels, whereas that of wild eels might be synchronized with behavioral and/or environmental
61 fluctuations that occur during the oceanic spawning migration.

62

63 **Keywords:** oocyte chorion, ultrastructure, wild adult eel, artificially matured eel, Japanese eel,

64 *Anguilla japonica*, spawning migration

65

66 **Introduction**

67 The Japanese eel *Anguilla japonica* is highly valued and is one of the most important aquaculture
68 species in Japan. Anguillid eels are catadromous fish that spawn offshore in the ocean, with the
69 juvenile growth phase occurring in freshwater [1]. These eels begin their downstream migration
70 toward the ocean for spawning at the onset of sexual maturation [2, 3]. Recently, stocks of wild glass
71 eels, which are used for aquaculture, have drastically decreased [4, 5], and this endangered species
72 appeared on the red list of the Ministry of the Environment of Japan in 2013. Therefore, the
73 establishment of effective techniques for the artificial production of glass eels is necessary to sustain a
74 seed supply and conserve this natural resource. Since Yamamoto and Yamauchi [6] first succeeded in
75 producing fertilized eggs and larvae by artificial hormone treatment, research on the artificial induction
76 of maturation and seed production in the Japanese eel has greatly increased [7–11]. Subsequently, the
77 production of second-generation larvae was achieved by artificial production in 2010 [12]. However,
78 one of the main obstacles to the mass production of glass eels is low egg quality, which leads to low
79 hatching and survival rates [13, 14]. To improve the artificial production of glass eels, the
80 physiological differences in oogenesis between wild and artificially maturing eels must be understood.

81 In the past, fully matured wild Japanese eels were not captured because the spawning sites of this
82 species are far from their growth habitats and were unidentified for many years [15]. Thus, no study
83 has yet described the natural oogenesis process that occurs during the spawning migration.
84 Immediately after the onset of downstream migration, the ovarian developmental stages of silver-phase
85 Japanese eels captured in rivers and coastal areas do not exceed the early vitellogenic stage [16, 17].
86 In contrast, silver-phase New Zealand longfinned eel *Anguilla dieffenbachii* and Celebes eel *Anguilla*
87 *celebesensis* have more developed ovaries at the mid-vitellogenic stage [18, 19]. Several studies have
88 conducted endocrinological and histological comparisons of oogenesis between naturally maturing
89 New Zealand longfinned eels and artificially maturing Japanese eels. In their endocrinological
90 research, Saito et al [20] indicated that the gonadotropin mRNA expression patterns of artificially
91 maturing Japanese eels differed from those of naturally maturing New Zealand longfinned eels.
92 Matsubara [21] showed that steroidogenic enzymes mRNA expression of artificially maturing Japanese
93 eels was overexpressed relative to that of naturally maturing New Zealand longfinned eels. In a
94 histological study, Lokman et al [22] showed that the chorion of naturally maturing New Zealand

95 longfinned eels was much thinner than that of artificially maturing Japanese eels, with that of the
96 mid-vitellogenic oocyte ranging between 350 and 450 μm .

97 The chorion is the outermost membrane of the egg. In teleosts, the chorion has two layers (zona
98 radiata externa, ZRE; and zona radiata interna, ZRI) that comprise the non-cellular envelope [e.g., 23,
99 24] and that function in the transport of needed materials during oocyte development [25]. In addition,
100 the chorion acts not only to prevent polyspermy but also to provide mechanical protection of the
101 embryo from external stimuli; thus, the chorion plays essential roles in fertilization and embryogenesis
102 [26–29]. Several studies have suggested that characteristics of the chorion influence the hatching
103 process in salmonids, including its hardness, structure and macromolecular composition [30–32].
104 Considering these findings and the previous report that the chorion of naturally maturing eels is much
105 thinner than that of artificially maturing eels [22], it is likely that chorion characteristics also influence
106 egg quality in the Japanese eel.

107 Ultrastructural investigations using transmission electron microscope (TEM) have demonstrated
108 that the ZRI of the chorion consists of several layers of alternating light and dark, electron-dense
109 structure at developing oocytes and ripe eggs of the artificially maturing Japanese eel [33, 34].
110 Adachi et al [35] suggested that the number of ZRI layers seems to correspond to the number of SPE
111 injections. Generally, the chorion proteins of teleosts are synthesized in the liver and/or ovaries [27,
112 28, 36]. In several species, such as salmonids and non-cyprinoid fish, chorion protein synthesis is
113 induced in the maternal liver by estrogen in the form of estradiol-17 β (E2) [37–41]. In the Japanese
114 eel, the levels of serum E2 exhibit a cyclic pattern, with large fluctuations occurring within one week of
115 SPE treatment [42]. Therefore, we hypothesized that chorion protein expression might also fluctuate
116 weekly with the E2 cyclic pattern induced by SPE injections, causing a series of alternating limited and
117 abundant protein accumulation during chorion formation. Therefore, the formation of the
118 electron-dense layer may be a phenomenon specific to artificially maturing eels in response to weekly,
119 repetitive injections. Furthermore, the thickness and ultrastructure of the chorion in artificially
120 maturing eels are likely abnormal, possibly contributing to low egg quality, however, this relationship
121 has yet to be established.

122 Recently, wild adult Japanese eels were captured in their spawning area for the first time [43–45],
123 allowing us to compare the reproductive physiology of Japanese eels matured in the wild and

124 artificially. Previously, we reported on the chorion thickness in wild adult Japanese eels, and our
125 results suggest that the chorion is slightly but significantly thinner than that of artificially matured eels
126 [45].

127 The aim of the present study was to clarify the detailed histological characteristics of the oocyte
128 chorion in wild adult and artificially matured eels and the differences in chorion characteristics between
129 them. First, we investigated the change in chorion thickness and volume during artificially induced
130 oogenesis. Then, we examined the relationship between the number of SPE injections and chorion
131 thickness. Next, chorion thickness was compared between artificially matured and wild eels.
132 Finally, chorion ultrastructure was observed using TEM.

133

134

135 **Materials and Methods**

136 *Animals*

137 Glass eels of Japanese eel were purchased from a commercial eel supplier in Japan and feminized
138 by perioral E2 administration (10 mg/kg diet) for 5 months. The eels were reared in freshwater
139 experimental tanks at the breeding facilities at the Faculty of Fisheries, Hokkaido University (Hakodate,
140 Hokkaido, Japan). The eels were fed commercial aquaculture feed ad libitum. Two-year-old
141 feminized eels were acclimated to seawater and received weekly injections of SPE (30 mg/kg body
142 weight) over 17 weeks to obtain maturing ovaries, following Chai et al [46]. Following anesthesia of
143 the eels in 2-phenoxyethanol, the developing ovaries were collected by abdominal surgery after 3–17
144 SPE injections. To obtain post-ovulatory ovaries and ovulated eggs, eels whose oocytes reached the
145 migratory nucleus stage after 12 or 13 SPE injections received $17\alpha, 20\beta$ -dihydroxy-4-pregnen-3-one
146 (DHP; 2 mg/kg body weight) injection to induce final maturation and ovulation, following Ohta et al
147 [7]. Forty nine artificially maturing eels (total length: 540–720 mm) and 4 artificially matured eels
148 (685–715 mm) were used in the present study.

149 Twelve wild adult Japanese eels were caught previously in the southern part of the West Mariana
150 Ridge [45]. Of these, 4 post-spawning females (Nos. 12–15 of Table 1 in Tsukamoto et al. [45]) were
151 used in the present study. The total lengths of the eels were 749, 767, 739 and 574 mm respectively.
152 All post-spawning females possessed ovaries, and most oocytes in the ovaries were at the

153 mid-vitellogenic stage. One female (No. 12) possessed over-ripened ovulated eggs within the
154 cavitas [45].

155 All experimental procedures complied with the National and Institutional Guidelines for the Care
156 and Use of Laboratory Animals and were approved by the Animal Research Committee of Hokkaido
157 University.

158

159 *Follicle diameter and chorion thickness*

160 Ovaries fixed in Bouin solution for 24 hours were transferred to 70% ethanol, dehydrated in an
161 ascending series of graded ethanol concentrations and embedded in paraffin. Sections 5 μm thick
162 were prepared and stained with hematoxylin and eosin. The sections were then observed under a
163 TUW-31-1 80i optical microscope (Nikon, Japan) and digitally photographed using a DXM 1200F
164 camera (Nikon, Japan). The following measurements were taken using ImageJ 1.47 software [47].
165 To ensure accurate measurement of the follicle diameter and chorion thickness, only undamaged
166 oocytes were selected. Oocytes ranging from the oil droplet stage to the migratory nuclear stage from
167 46 artificially maturing eels (6 oocytes per eel) after 3–12 SPE injections were examined to determine
168 changes in chorion thickness and volume during artificially induced oogenesis. In addition, oocytes
169 ranging between 380 and 420 μm obtained from 9 artificially maturing eels (3–6 oocytes per eel) at 6–
170 17 SPE injections were examined to determine a relationship between the number of SPE injections
171 and chorion thickness. For the wild post-spawning eels (Nos. 12–15), 350–600 μm oocytes were
172 most abundant in their post-ovulatory ovaries [45]. Thus, to minimize the effect of differences in
173 oocyte size, we used only oocytes of the post-ovulatory ovary, which had diameters of between 380 μm
174 and 420 μm , to make comparisons between the wild and artificially matured eels. Oocytes from the 4
175 wild and 4 artificially matured eels (16 oocytes per eel) were examined. Subsequently, the chorion
176 volume ($\text{CV}, \mu\text{m}^3$) was calculated from the follicle diameter ($\text{FD}, \mu\text{m}$) and chorion thickness ($\text{CT}, \mu\text{m}$)
177 as follows:

$$178 \quad \text{CV} = \pi [\text{FD}^3 - (\text{FD} - 2\text{CT})^3] / 6$$

179

180 *Electron microscope observations*

181 Sections of ovaries and eggs were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1
182 M cacodylate buffer (pH 7.4) for 24 hours at 4°C. After washing in cacodylate/sucrose buffer (pH
183 7.4), the sections were post-fixed in 1% osmium tetroxide in the cacodylate buffer for 2.5 hours at 4°C.
184 The sections were then dehydrated and embedded in EPON 812 (TAAB Laboratories Equipment, UK).
185 Ultrathin sections (approximately 70 nm) were prepared and stained with 2.5% samarium acetate and
186 2.66% lead citrate and observed using a JEM-1011 TEM (JEOL, Japan) equipped with an iTEM digital
187 camera (Olympus, Germany).

188

189 *Statistical analyses*

190 Pearson's coefficient tests using log-transformed values were carried out to test for relationships
191 between follicle diameter and chorion thickness, follicle diameter and chorion volume and chorion
192 thickness and the number of SPE injections. To compare the chorion thickness between wild and
193 artificially matured eels, Kruskal-Wallis tests, followed by Scheffe's tests, were conducted. Variables
194 are expressed as the mean \pm S.D., and significant differences were calculated at $P < 0.05$. Statistical
195 analyses were performed using Excel statistical Analysis 2012 (SSRI, Japan).

196

197

198 **Results**

199 *Change in chorion thickness and volume during artificially induced oogenesis*

200 Chorion thickness increased linearly with ovarian follicle development until follicle diameter
201 reached approximately 450 μm . It then began to decrease with further increases in follicle diameter
202 (450–900 μm) (Fig. 1). A statistically significant positive correlation between chorion thickness and
203 follicle diameter was found within the 200–450 μm range in follicle diameter ($R^2 = 0.83$, $P < 0.001$),
204 whereas within the 450–900 μm range, a significant negative correlation was observed ($R^2 = 0.70$, $P <$
205 0.001).

206 The chorion volume increased with ovarian follicle development, peaking at approximately 450
207 μm (Fig. 2), as did chorion thickness (Fig. 1). A significant positive correlation between chorion
208 volume and follicle diameter within the 200–450 μm range ($R^2 = 0.93$, $P < 0.001$) was found.

209 However, no clear relationship between chorion volume and follicle diameter was observed in the
210 450–900 μm range ($R^2 = 0.02$, $P \geq 0.05$).

211

212 ***Relationship between the number of SPE injections and chorion thickness***

213 As chorion thickness varied with follicle diameter during oogenesis in artificially maturing eels
214 (Fig. 1), the relationship between the number of SPE injections and chorion thickness was examined
215 only in those oocytes within the same range of follicle diameter (380–420 μm). A significant positive
216 correlation between the number of SPE injections and chorion thickness of 380–420 μm follicles was
217 detected in artificially maturing eels ($R^2 = 0.79$, $P < 0.01$; Fig. 3).

218

219 ***Chorion thickness in wild and artificially matured eels***

220 Residual follicles in post-ovulatory ovaries of artificially ovulated eels were used for comparison
221 with wild eels because wild adult females were in post-spawning condition. The chorion thicknesses
222 of 380–420 μm oocytes in the post-ovulatory ovaries of wild post-spawning and artificially matured
223 eels are shown in Figure 4. In wild post-spawning eels, the chorion thickness of No. 12 (6.85 ± 0.62
224 μm) was significantly greater than those of the remaining three wild eels (No. 13: 3.88 ± 0.50 μm ; No.
225 14: 4.56 ± 0.48 μm ; No. 15: 3.85 ± 0.62 μm) ($P < 0.01$), which varied among individuals. In contrast,
226 there was no significant difference in chorion thickness among the four artificially matured eels (No. 1:
227 6.12 ± 0.93 μm ; No. 2: 5.69 ± 0.35 μm ; No. 3: 5.38 ± 0.52 μm ; No. 4: 5.34 ± 0.82 μm) ($P \geq 0.05$).
228 The chorion thicknesses of two wild eels (No.13 and No.15) were significantly thinner than those of
229 three artificially matured eels (Nos. 1, 2 and 3) ($P < 0.05$).

230

231 ***Chorion ultrastructure in wild and artificially matured eels***

232 The ultrastructure of the oocyte chorion of post-ovulatory ovaries and ovulated eggs in wild
233 post-spawning eels and artificially matured eels are shown in Figure 5. The chorion consisted of two
234 layers: a thinner ZRE and a thicker ZRI. The ZRI had several layers that alternated light and dark
235 uniform electron-dense. Among the wild post-spawning eels, the oocyte chorion of the post-ovulatory
236 ovaries in No. 12 had eight dense layers (Fig. 5a) and the remaining three wild eels had six dense layers
237 (Fig. 5 b, c, d). Similarly, the oocyte chorion of post-ovulatory ovaries in artificially matured eel at 13

238 SPE injections had six dense layers (Fig. 5e). The ovulated egg in No. 12 also had eight dense layers,
239 as did the oocyte of post-ovulatory ovaries in same individual (Fig. 5a).

240

241

242 **Discussion**

243 *Changes in chorion thickness and volume during oogenesis*

244 Our results showed that changes in chorion thickness occurred with ovarian follicle diameter
245 development in artificially maturing eels. A similar pattern of chorion change has also been observed
246 in the whitespotted conger *Conger myriaster* and the large yellow croaker *Pseudosciaena crocea* [48,
247 49]. Based on ultrastructure observations, Kayaba et al [34] reported that the chorion thickness of
248 Japanese eels increased from the oil droplet stage to the vitellogenic stage. Moreover, Oka [50]
249 showed that the chorion thickness of Japanese eels increased from the oil droplet stage to the secondary
250 yolk stage and decreased from the tertiary yolk stage to the migratory nucleus stage. Additionally, we
251 investigated the change in chorion volume during artificially induced oogenesis and found that the
252 chorion volume also increased with increasing follicle diameter, peaking at 450 μm ; however, no
253 significant change was observed beyond that point. Previously, northern blot analysis showed that the
254 chorion protein genes *zpb* and *zpc* in the ovary of the Japanese eel decreased simultaneously with
255 oogenesis [51, 52]. Furthermore, using quantitative real time RT-PCR, mRNA expression of *zpb* and
256 *zpc* in the European eel *Anguilla anguilla* was found to decrease from the mid-vitellogenic stage to
257 the late-vitellogenic stage [53]. Considering these findings together, we suggest that formation of
258 the chorion may cease after the mid-vitellogenic stage, with the chorion stretching and consequently
259 becoming thinner with increasing follicle diameter.

260

261 *Relationship between the number of SPE injections and chorion thickness*

262 This study is the first report on the relationship between the number of SPE injections and chorion
263 thickness, suggesting that chorion thickness likely varies with the number of SPE injections received in
264 the artificially maturing eel. A significant positive correlation between the number of SPE injections
265 and chorion thickness was observed at follicle diameters of 380–420 μm . Moreover, the chorion
266 thickness of a 400 μm oocyte of artificially maturing eel (first batch of developing follicles), as

267 calculated from the regression formula ($4.73 \mu\text{m}$; Fig. 1), is thinner than that of oocytes in ovaries after
268 ovulation in the four artificially matured eels (second batch of follicles, for which more SPE injections
269 were received than the first batch). Chorion proteins are synthesized by E2 stimulation in the liver in
270 rainbow trout *Oncorhynchus mykiss*, Medaka *Oryzias latipes*, masu salmon *Oncorhynchus masou*,
271 Sakhalin taimen *Hucho perryi* and gilthead sea bream *Sparus aurata* [37–41]. Observed annual
272 changes in serum chorion protein levels are similar to those of serum E2 levels in Sakhalin taimen and
273 masu salmon [39, 54, 55]. In addition, the serum E2 levels of Japanese eel have been observed to
274 increase following SPE treatment [42]. Although E2-dependent chorion proteins have not yet been
275 identified in Japanese eels, considering the above findings, we suppose that SPE injections facilitate
276 chorion formation during artificial maturation.

277

278 ***Chorion thickness in wild and artificial eels***

279 In a preliminary report, we noted that the chorion of wild eels appears to be significantly thinner
280 than that of artificially matured eels; however, the number of examined oocytes was insufficient for
281 detailed comparison [45]. Furthermore, we did not evaluate the variation in chorion thickness among
282 wild eels, and changes in chorion thickness with increasing follicle diameter were not investigated.
283 Therefore, this study is the first strict comparison of chorion thickness between wild and artificially
284 matured eels. Chorion thicknesses varied among wild eels, and those of two wild eels (No. 13 and No.
285 15) were significantly thinner than those of the artificially matured eels. Additionally, the chorion of
286 an additional wild eel (No. 14) was thinner, although not significantly so, than those of artificially
287 matured eels. This result is in agreement with a previous report that the chorion of maturing wild
288 New Zealand longfinned eels appeared to be much thinner than those of artificially maturing Japanese
289 eels at 350–450 μm follicle diameters [22]. However, the chorions of No. 12 were significantly
290 thicker than those of the remaining 3 wild eels and almost equally thick as those of artificially matured
291 eels. It is possible that No. 12 experienced a different environment, route and/or distance to the other
292 3 eels during their spawning migration, although there is no evidence to support this.

293 A possible reason why the chorions of artificially matured eels were generally thicker than those
294 of wild eels may be the SPE injections. The levels of the serum E2 cycle, with large fluctuations
295 occurring within one week of SPE treatment in Japanese eels [42]. With the rapid increase in serum

296 E2, chorion protein was also over-synthesized; consequently, the chorion may have thickened. In the
297 present study, the chorion thickness of one wild eel was similar to that of the artificially matured eels;
298 therefore, it is not possible to conclude that chorion thickness influences egg quality. However, the
299 positive correlation between the number of weekly SPE injections and chorion thickness suggests that
300 improvements in SPE injection methods lead to the production of eggs morphologically similar to wild
301 eggs; such improvements may lead to advances in artificial seed production in Japanese eels.

302

303 *Chorion ultrastructure in wild and artificially matured eels*

304 This study is the first study of chorion ultrastructure in wild post-spawning eels. We found that
305 the ZRI of post-ovulatory ovaries and ovulated eggs consisted of several layers of alternating light and
306 dark, electron-dense structure in wild post-spawning eels as well as artificially matured eels. As a
307 similar structure was reported in artificially maturing eels in previous studies [33, 34], we had
308 hypothesized that the formation of the electron-dense layer is particular to artificially maturing eels,
309 owing to repeated SPE injections. However, our results are not consistent with this hypothesis,
310 suggesting that the layer structure of the ZRI is common to both wild and artificially maturing eels.

311 Although similar layers have also been observed in other species, such as Medaka, the marbled
312 swamp eel *Synbranchus marmoratus*, Atlantic bluefin tuna *Thunnus thynnus* and the gilthead seabream,
313 the formative factor of the layer and structural difference between the dense and common layers is
314 poorly understood [23, 56–58]. A previous study of artificially maturing eels reported that the
315 number of ZRI layers appears to correspond with the number of SPE injections received [35]. Thus,
316 we suspect that the fluctuation in blood E2 level induced by the weekly SPE injections caused the
317 repeated pattern of limited and abundant protein accumulation during chorion formation, resulting in
318 the formation of the electron-dense layer. However, the present study demonstrated an inconsistency
319 between the number of SPE injections and the number of layers; for example, the oocyte chorion of
320 ovaries after ovulation in an artificially matured eel that received 13 SPE injections had six dense
321 layers. This inconsistency may be because the oocyte was at the oil droplet stage before receiving
322 SPE injections, a stage potentially too early for chorion accumulation. It was previously observed that
323 the formation of the ZRI was initiated after the oocyte reached the early vitellogenic stage [34].
324 Therefore, the first several SPE injections may not have contributed to layer formation.

325 In contrast, the ZRI of wild eels had the electron-dense layer, suggesting that the chorion
326 formation cycle in wild eels is synchronized with behavioral and/or environmental fluctuations during
327 their oceanic spawning migration. One possible environmental factor that appears to affect chorion
328 formation is the lunar cycle. A recent bio-logging study found an obvious impact of the lunar cycle
329 on the upper limit of migration depth in the tropical eel *Anguilla marmorata* [59]. We reviewed the
330 bio-logging data of Jellyman and Tsukamoto [60], which include the depth and temperature profiles of
331 the temperate eel *Anguilla dieffenbachii* during the oceanic spawning migration. We found that the
332 swimming depths during the full moon period were deeper than those during the new moon and that the
333 empirical temperatures at the full moon were lower than those at the new moon (Fig. 6). Furthermore,
334 a bio-logging study of Japanese eels also showed similar behavioral patterns, i.e., that swimming
335 depths during the full moon were deeper than those during the new moon during oceanic migration
336 (Watanabe S, pers. comm., 2013). The similar behavioral response of a tropical eel and a temperate
337 eel to the lunar cycle suggests that this behavior is common to anguillid eels and that they may
338 experience monthly temperature fluctuations during the oceanic spawning migration. Several
339 previous reports have suggested that steroidogenesis is affected by changes in water temperature in
340 female eels [61–63]. Considering all these findings, we hypothesize that chorion formation is
341 accelerated at higher temperatures during the new moon and delayed at lower temperature during the
342 full moon, consequently forming electron-dense layers with a circalunar rhythm.

343 Wild eel No. 12 had a thick chorion with many layers relative to the other 3 wild eels. Assuming
344 that the electron-dense layers form synchronously with the lunar cycle, the period of spawning
345 migration may have been longer for No. 12 than for the other 3 wild eels. The Japanese eel is
346 distributed throughout Taiwan, China, the Korean Peninsula and Japan [15]. These geographic
347 differences may influence individual variability during the spawning migration.

348 In this study, we provide the first description of the histological characteristics of the oocyte
349 chorion in wild post-spawning and artificially matured Japanese eels. The chorion thickness of
350 artificially induced eels was positively correlated with the number of SPE injections received. The
351 chorion thicknesses of two wild eels were significantly thinner than those of artificially matured eels.
352 However, the influence of chorion thickness on egg quality remains unclear, and more experimental
353 comparisons are needed. Further research on the reproductive physiology and biology of artificially

354 matured and wild adult eels is needed to reveal the natural processes of oocyte development, and this
355 information may lead to advances in the production of high-quality eggs in artificially matured eels.

356

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366

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538

539 **Figure Captions**

540

541 **Fig. 1** Changes in chorion thickness during artificially induced oogenesis ($n = 276$). The dashed
542 line indicates the breakpoint at 450 μm follicle diameter

543

544 **Fig. 2** Changes in chorion volume during artificially induced oogenesis ($n = 276$). The dashed line
545 indicates the breakpoint at 450 μm follicle diameter

546

547 **Fig. 3** Relationship between the number of SPE injections and chorion thickness within oocytes of
548 380–420 μm follicle diameter in artificially maturing Japanese eels

549

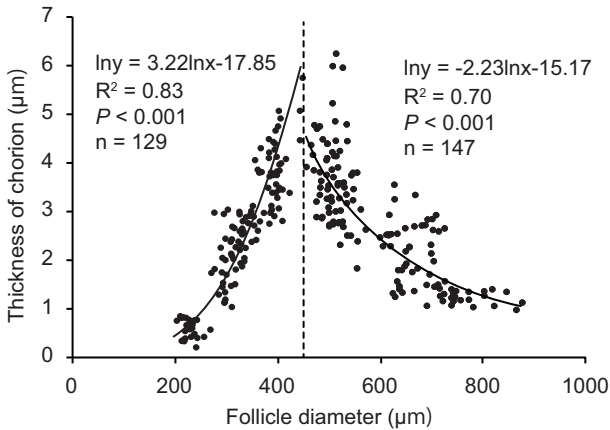
550 **Fig. 4** Box plots of chorion thickness of 380–420 μm oocytes from post-ovulatory ovaries of
551 post-spawning wild and artificially matured Japanese eels. The top and bottom of the boxes are the
552 upper and lower quartiles, and the line in each box is the median. The ends of the whiskers indicate
553 the lowest/highest datum still within the 1.5 interquartile range of the lower/upper quartile. Different
554 letters indicate significant differences among individuals ($P < 0.05$)

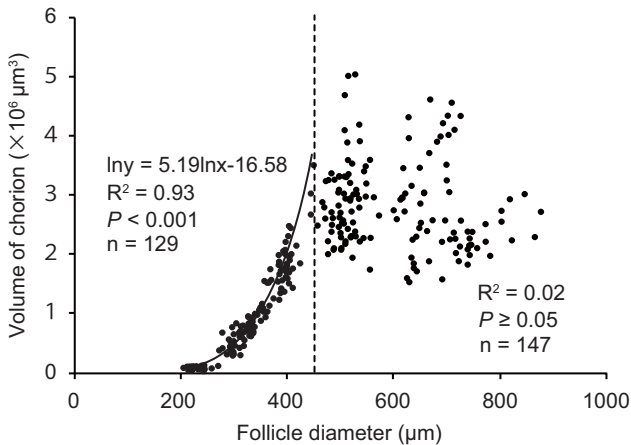
555

556 **Fig. 5** Ultrastructure of the oocyte chorion in wild and artificially matured Japanese eels. (a)
557 Post-ovulatory ovarian follicle of No. 12, (b) No. 13, (c) No. 14, (d) No. 15 and (e) an artificially
558 matured eel. The zona radiata interna (ZRI) of the oocyte of No. 12 had eight layers (alternating dark
559 and light bands); six layers were observed in the remaining three wild females (No. 13, No. 14 and No.
560 15). (f) Ovulated egg in No. 12. The ZRI of the egg of No. 12 had eight layers. Scale bars indicate
561 2 μm

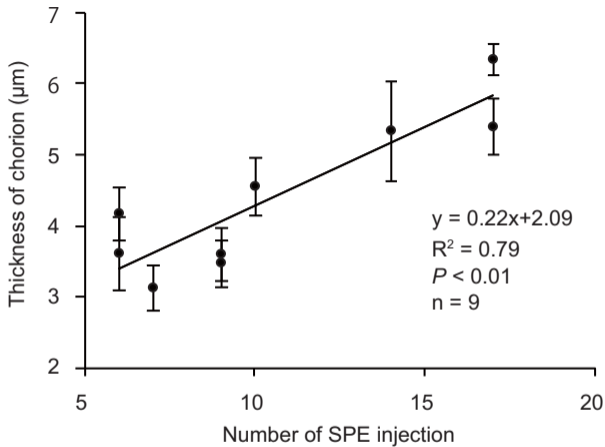
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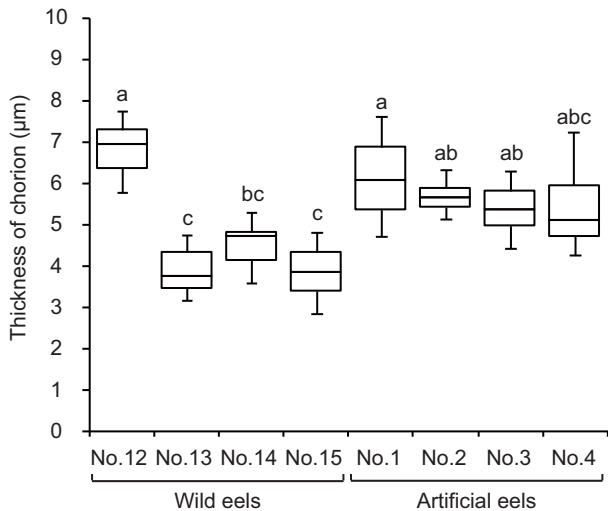
563 **Fig. 6** Relationship between lunar cycle and nighttime water temperature in 3 individual New
564 Zealand longfin eels *Anguilla dieffenbachii* during their oceanic spawning migration (modified from
565 Fig. 1 in Jellyman and Tsukamoto. [60]). Filled symbols: average of a complete 12 h dataset; unfilled
566 symbols: average of a 6 h dataset. Dashed lines: days of full moon; solid lines: days of new moon.
567 Arrows labeled Eel 1, 2 and 3 indicate the end of the liberty periods (see Jellyman and Tsukamoto.
568 [60])



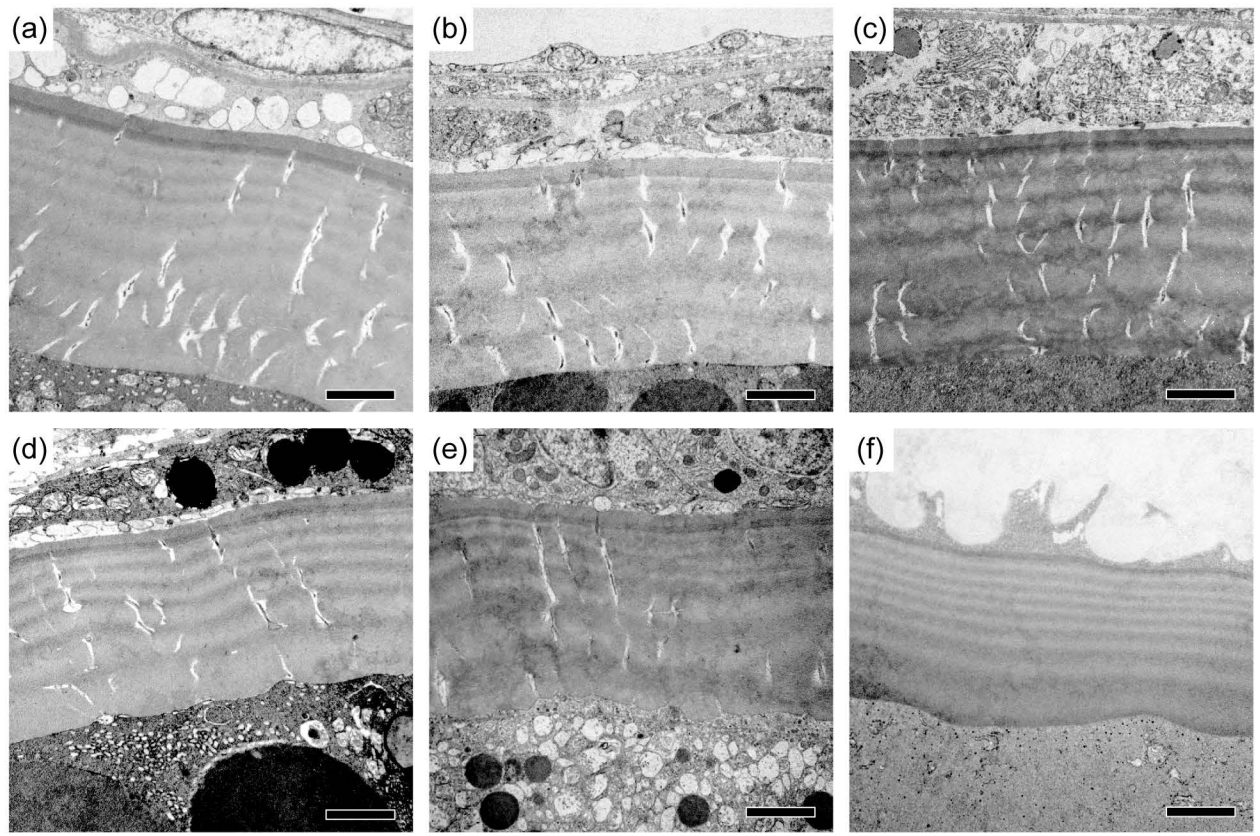


Izumi et al., Fig. 2

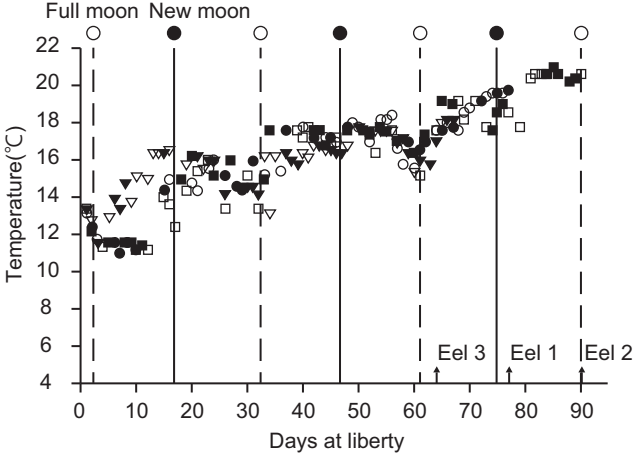




Izumi et al., Fig. 4



Izumi et al., Fig. 5



Izumi et al., Fig. 6