Histological characteristics of the oocyte chorion in wild post-spawning and artificially matured Japanese eels *Anguilla japonica*

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Abstract

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

Keywords: oocyte chorion, ultrastructure, wild adult eel, artificially matured eel, Japanese eel, *Anguilla japonica*, spawning migration

Introduction

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

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

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

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

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

Recently, wild adult Japanese eels were captured in their spawning area for the first time [43–45], 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 17α, 20β-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 (CV, μm^3) was calculated from the follicle diameter (FD, μm) and chorion thickness (CT, μm)
177 as follows:
178 $$CV = \pi [FD^3 - (FD - 2CT)^3] / 6$$
179
180 ***Electron microscope observations***

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

Statistical analyses

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

Results

Change in chorion thickness and volume during artificially induced oogenesis

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

The chorion volume increased with ovarian follicle development, peaking at approximately 450 µm (Fig. 2), as did chorion thickness (Fig. 1). A significant positive correlation between chorion 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

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

Discussion

Changes in chorion thickness and volume during oogenesis

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

Relationship between the number of SPE injections and chorion thickness

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

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

Chorion thickness in wild and artificial eels

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

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

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

Chorion ultrastructure in wild and artificially matured eels

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

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

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

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

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

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

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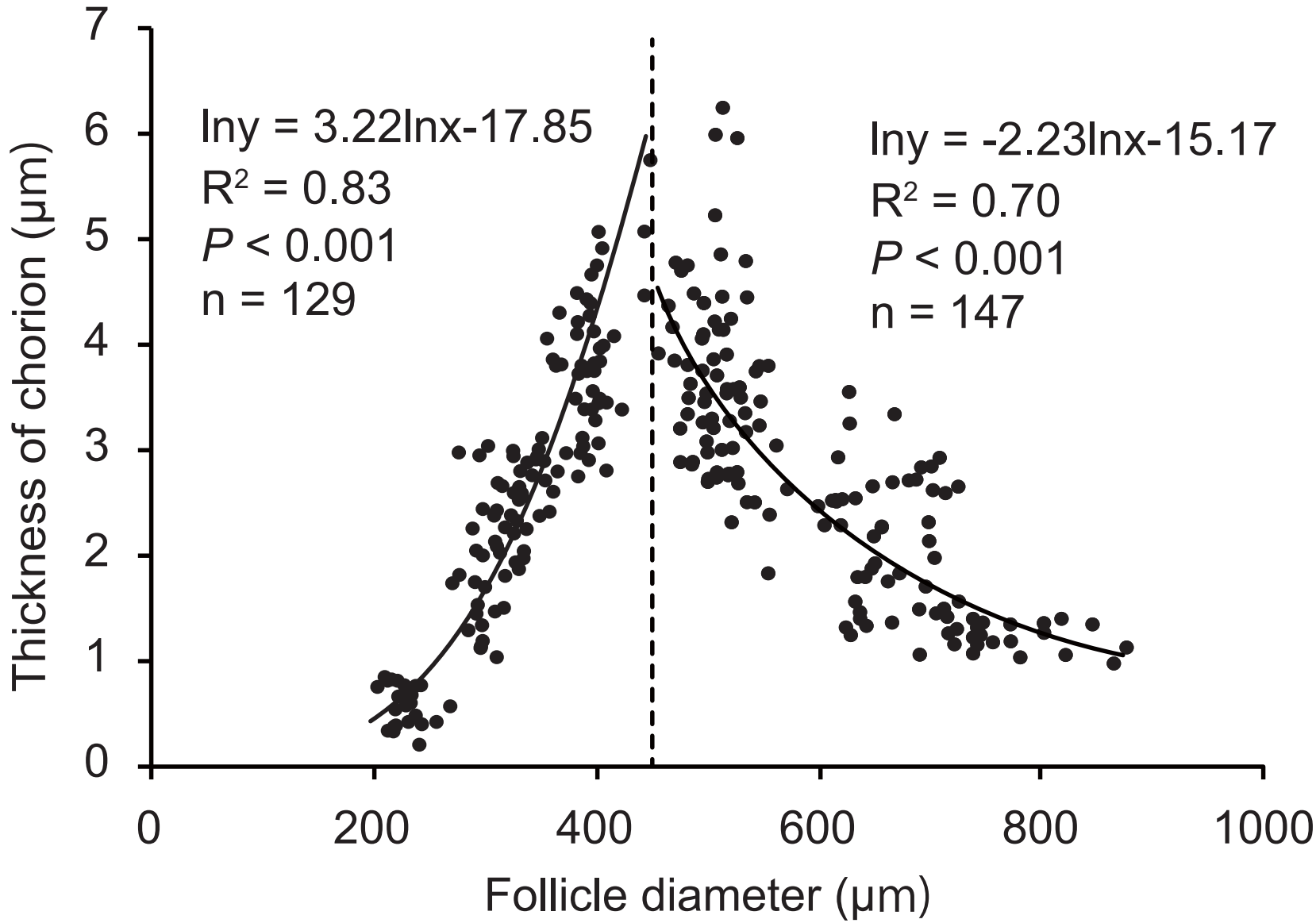
Figure Captions

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

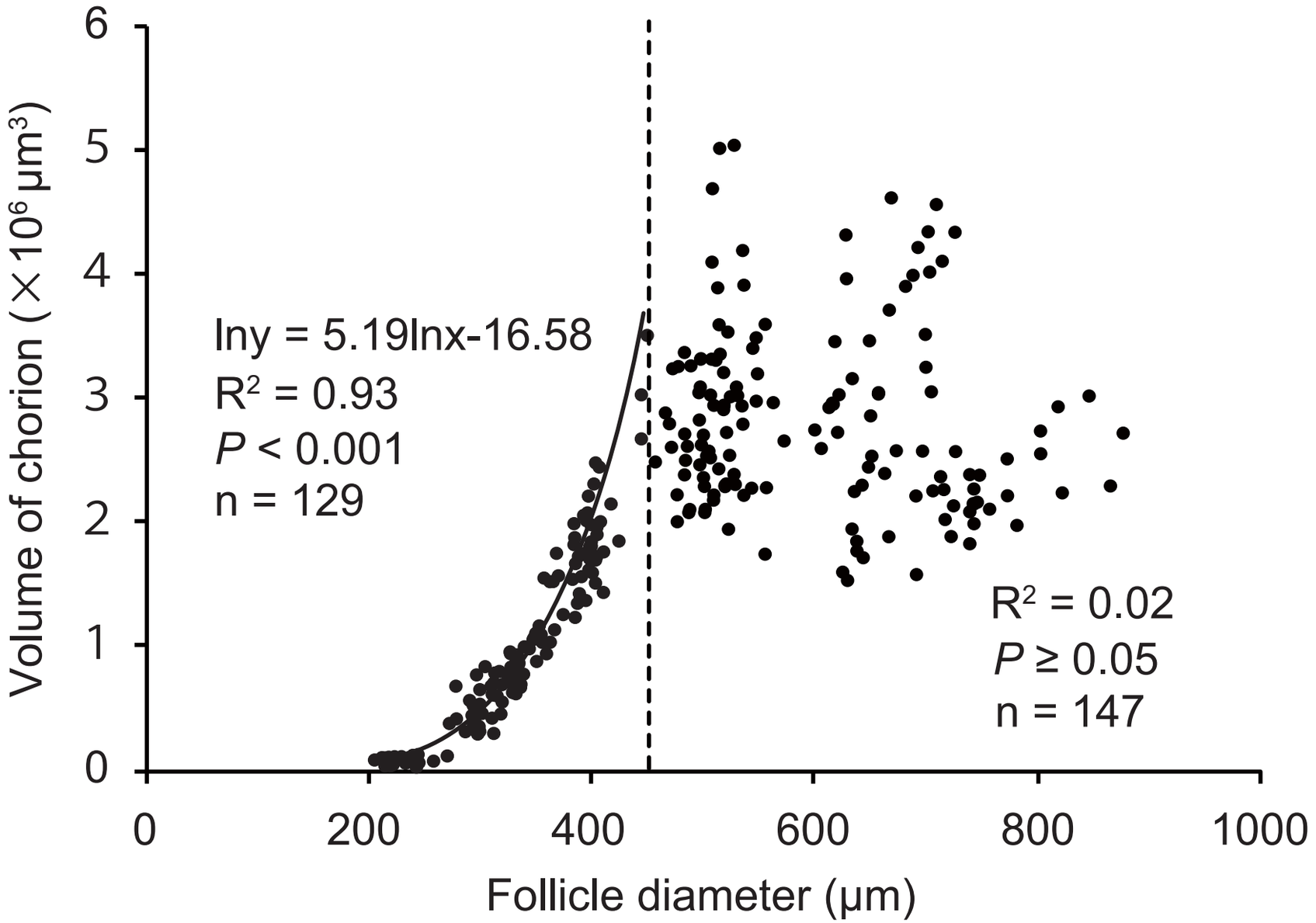
Fig. 2 Changes in chorion volume during artificially induced oogenesis (n = 276). The dashed line indicates the breakpoint at 450 μm follicle diameter

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

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
562
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. 1



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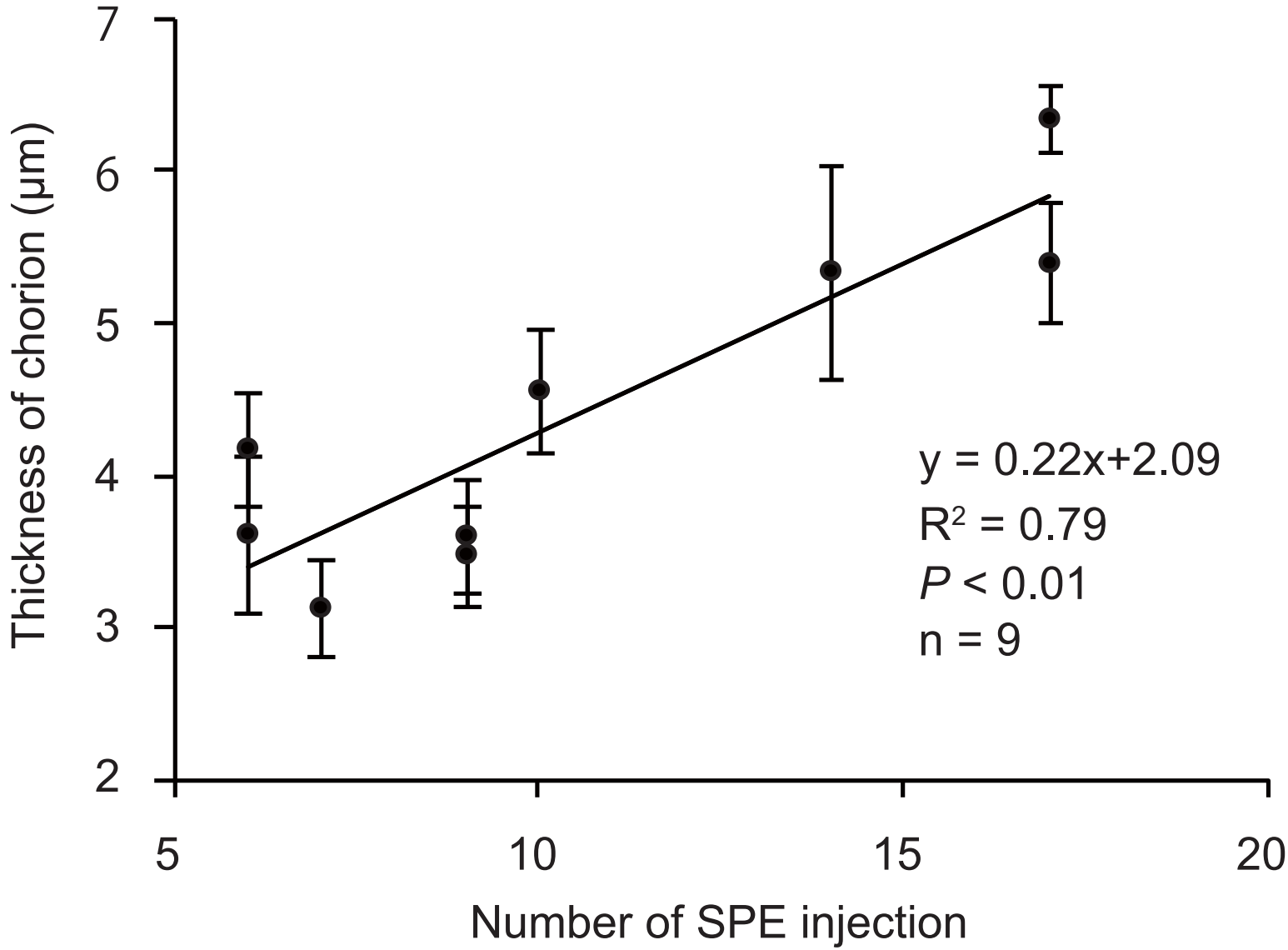

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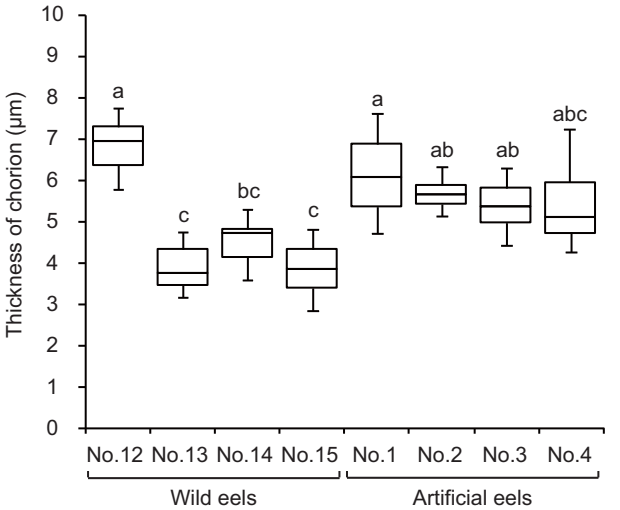

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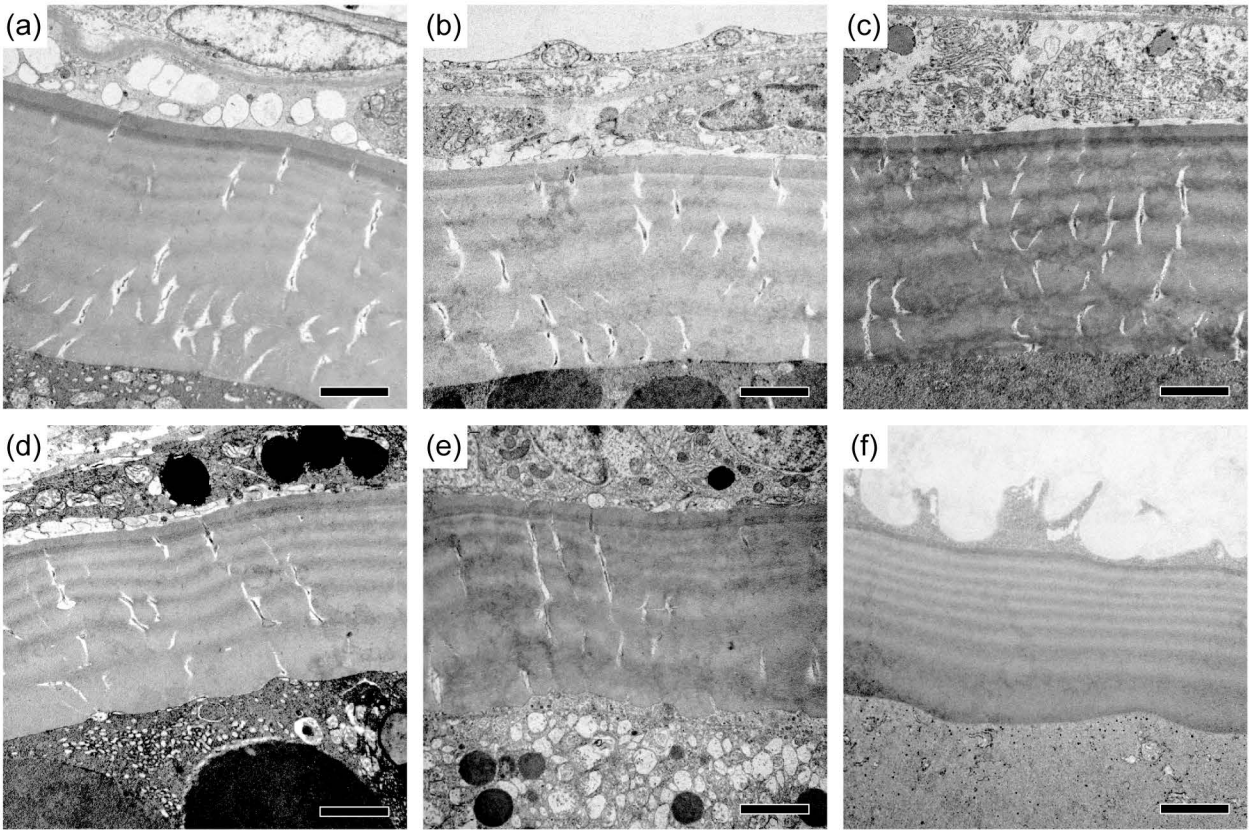

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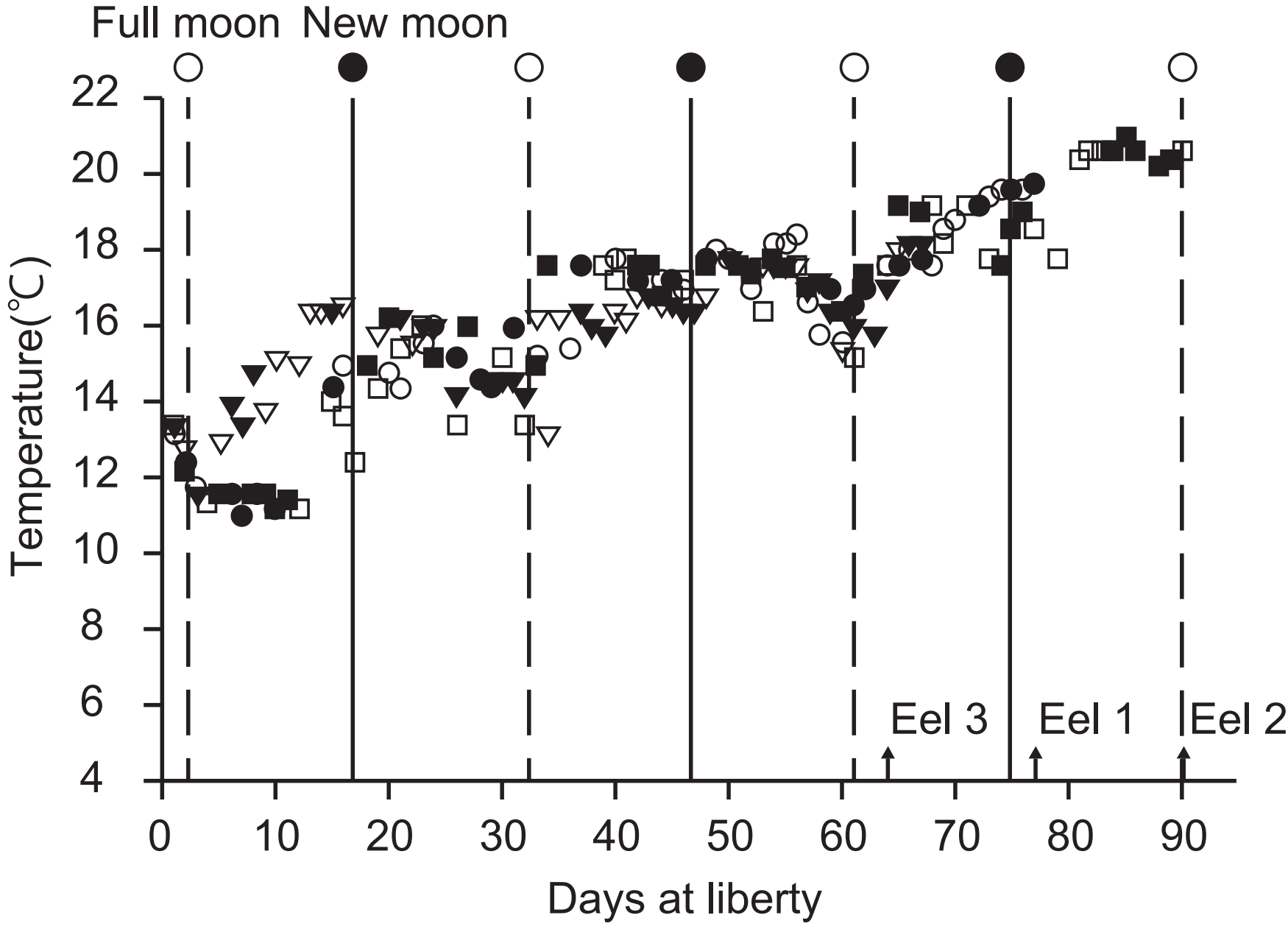

Izumi et al., Fig. 6