Fish Sci
DOI 10.1007/s12562-015-0851-1

ORIGINAL ARTICLE **Biology**

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

Hikari Izumi · Seishi Hagihara · Hiroaki Kurogi · Seinen Chow · Katsumi Tsukamoto · Hirohiko Kagawa · Hideaki Kudo · Shigeho Ijiri · Shinji Adachi

Received: 24 July 2014 / Accepted: 21 December 2014

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 matured eels. 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 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

H. Izumi · S. Hagihara · S. Ijiri (✉) · S. Adachi
Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
e-mail: ijiri@fish.hokudai.ac.jp

H. Kurogi
Yokosuka Laboratory, National Research Institute of Aquaculture, Fisheries Research Agency, Yokosuka, Kanagawa 238-0316, Japan

S. Chow
National Research Institute of Fisheries Science, Fisheries Research Agency, Yokohama, Kanagawa 236-8648, Japan

K. Tsukamoto
Department of Marine Science and Resources, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-0880, Japan

H. Kagawa
Department of Marine Biology and Environmental Sciences, Faculty of Agriculture, University of Miyazaki, Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan

H. Kudo
Division of Marine Bioresource and Environmental Science, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

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 fresh water [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 artificial production of glass eels is necessary to sustain a seed supply

Published online: 08 February 2015

and conserve this natural resource. Since Yamamoto and Yamauchi [6] first succeeded in producing fertilized eggs and larvae by artificial hormone treatment, research on artificial induction of maturation and seed production in the Japanese eel has greatly increased [7–11]. Subsequently, in 2010, artificial production of second-generation larvae was achieved [12]. However, one of the main obstacles to mass production of glass eels is low egg quality, which leads to low hatching and survival rates [13, 14]. To improve 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 messenger RNA (mRNA) expression patterns of artificially maturing Japanese eels differed from those of naturally maturing New Zealand longfinned eels. Matsubara [21] showed that mRNA expression of steroidogenic enzymes in artificially maturing Japanese eels was overexpressed relative to that in 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 form the noncellular envelope, e.g., [23, 24], and that function in the transport of materials necessary 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 microscopy (TEM) have demonstrated that the ZRI of the chorion consists of several layers of alternating light and dark, electron-dense structure for 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 with 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 noncyprinoid 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 artificially. Previously, we reported on the chorion thickness in wild adult Japanese eels, and our results suggest that the chorion is slightly but significantly thinner than that of artificially matured eels [45].

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

Materials and methods

Animals

Glass eels of Japanese eel were purchased from a commercial eel supplier in Japan and feminized by peroral E2

administration (10 mg/kg diet) for 5 months. The eels were reared in freshwater experimental tanks at the breeding facilities at the Faculty of Fisheries, Hokkaido University (Hakodate, Hokkaido, Japan). The eels were fed commercial aquaculture feed ad libitum. Two-year-old feminized eels were acclimated to sea water and received weekly injections of SPE (30 mg/kg body weight) over 17 weeks to obtain maturing ovaries, following Chai et al. [46]. Following anesthesia of the eels in 2-phenoxyethanol, the developing ovaries were collected by abdominal surgery after 3–17 SPE injections. To obtain post-ovulatory ovaries and ovulated eggs, eels whose oocytes reached the migratory nucleus stage after 12 or 13 SPE injections received 17α,20β-dihydroxy-4-pregnen-3-one (DHP; 2 mg/kg body weight) injection to induce final maturation and ovulation, following Ohta et al. [7]. Forty-nine artificially maturing eels (total length 540–720 mm) and 4 artificially matured eels (685–715 mm) were used in the present study.

Twelve wild adult Japanese eels were caught previously in the southern part of the West Mariana Ridge [45]. Of these, 4 post-spawning females (nos. 12–15 of Table 1 in Tsukamoto et al. [45]) were used in the present study. The total lengths of the eels were 749, 767, 739, and 574 mm, respectively. All post-spawning females possessed ovaries, and most oocytes in the ovaries were at the mid-vitellogenic stage. One female (no. 12) possessed over-ripened ovulated eggs within the cavitas [45].

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

Follicle diameter and chorion thickness

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

$$CV = \pi\ [FD^3 - (FD-2CT)^3]/6.$$

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 h 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 h 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 chorion thickness between wild and artificially matured eels, Kruskal–Wallis tests, followed by Scheffé's tests, were conducted. Variables are expressed as mean ± standard deviation (SD), 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

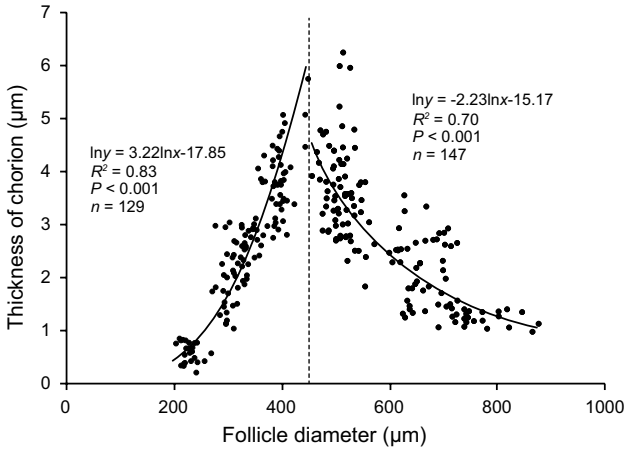

Fig. 1 Changes in chorion thickness during artificially induced oogenesis ($n = 276$). The *dashed line* indicates the breakpoint at 450 μm follicle diameter

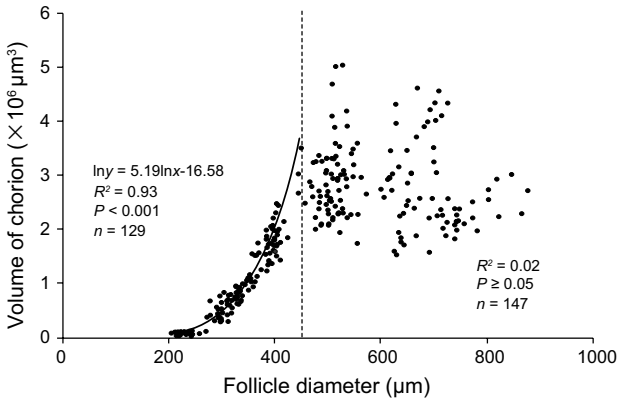

Fig. 2 Changes in chorion volume during artificially induced oogenesis ($n = 276$). The *dashed line* indicates the breakpoint at 450 μm follicle diameter

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

Relationship between the number of SPE injections and chorion thickness

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

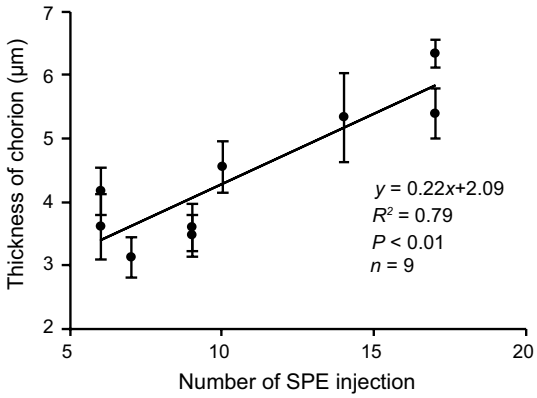

Fig. 3 Relationship between the number of SPE injections and chorion thickness within oocytes of 380–420 μm follicle diameter in artificially maturing Japanese eels

Chorion thickness in wild and artificially matured eels

Residual follicles in post-ovulatory ovaries of artificially ovulated eels were used for comparison with wild eels because wild adult females were in post-spawning condition. The chorion thicknesses of 380–420 μm oocytes in the post-ovulatory ovaries of wild post-spawning and artificially matured eels are shown in Fig. 4. In wild post-spawning eels, the chorion thickness of no. 12 (6.85 ± 0.62 μm) was significantly greater than those of the remaining three wild eels (no. 13: 3.88 ± 0.50 μm; no. 14: 4.56 ± 0.48 μm; no. 15: 3.85 ± 0.62 μm) ($P < 0.01$), which varied among individuals. In contrast, there was no significant difference in chorion thickness among the four artificially matured eels (no. 1: 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) ($P \geq 0.05$). The chorion thicknesses of two wild eels (no. 13 and no. 15) were significantly thinner than those of three artificially matured eels (nos. 1–3) ($P < 0.05$).

Chorion ultrastructure in wild and artificially matured eels

The ultrastructure of the oocyte chorion of post-ovulatory ovaries and ovulated eggs in wild post-spawning eels and artificially matured eels are shown in Fig. 5. The chorion consisted of two layers: a thinner ZRE and a thicker ZRI.

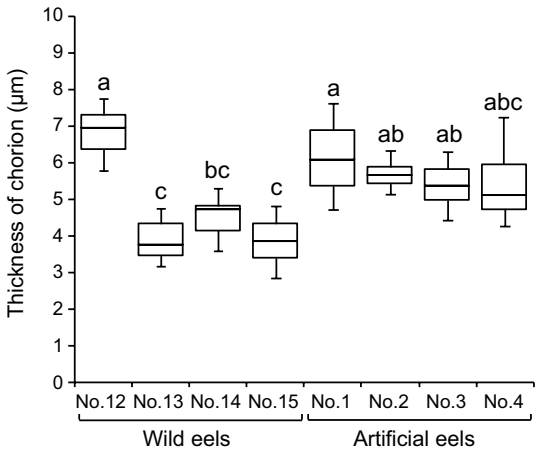

Fig. 4 *Box plots* of chorion thickness of 380–420 μm oocytes from post-ovulatory ovaries of post-spawning wild and artificially matured Japanese eels. The *top and bottom of the boxes* are the upper and lower quartiles, and the *line in each box* is the median. The *ends of the whiskers* indicate the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

The ZRI had several layers that alternated light and dark uniform electron-dense. Among the wild post-spawning eels, the oocyte chorion of the post-ovulatory ovaries in no. 12 had eight dense layers (Fig. 5a) while the remaining three wild eels had six dense layers (Fig. 5b–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 the 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*

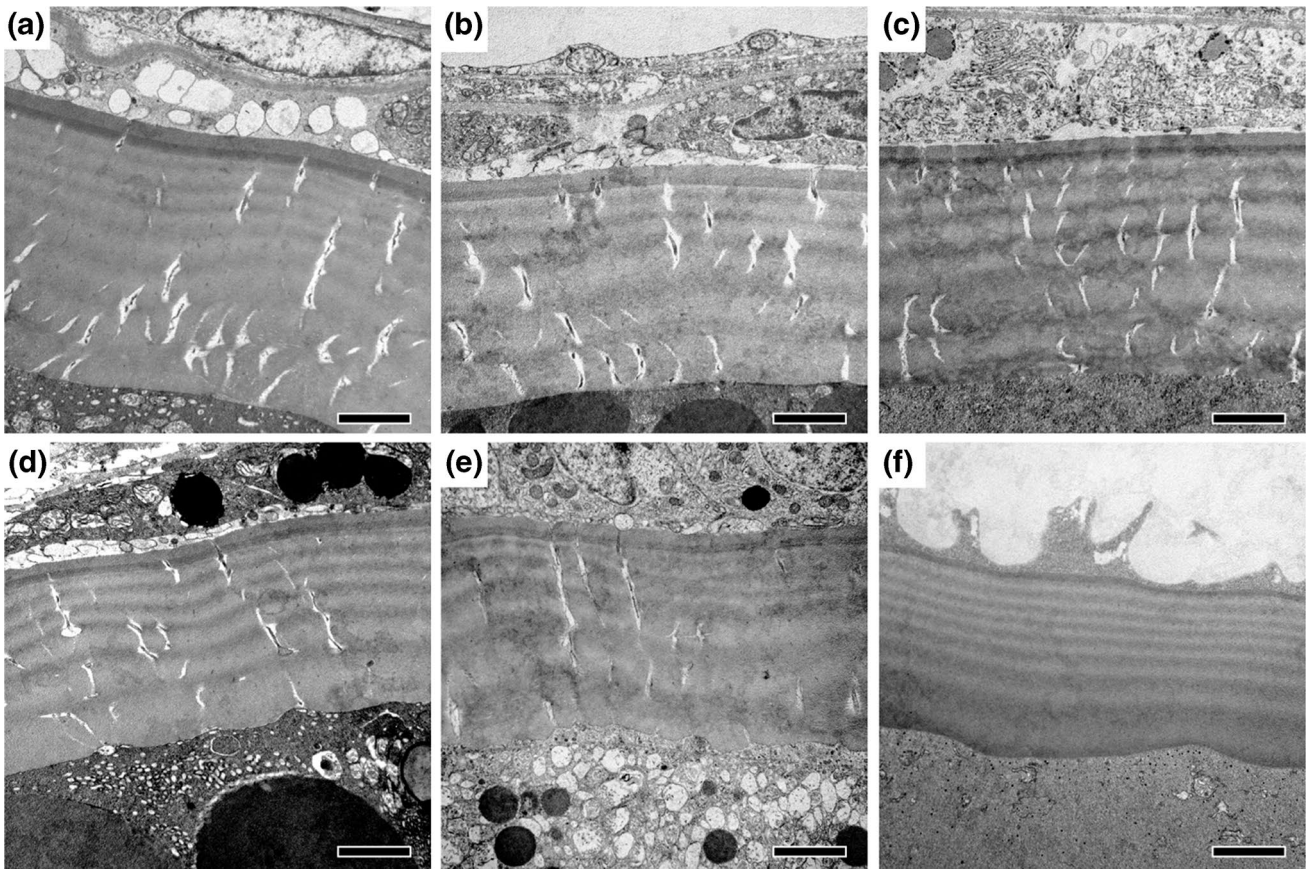

Fig. 5 Ultrastructure of the oocyte chorion in wild and artificially matured Japanese eels. **a** Post-ovulatory ovarian follicle of no. 12, **b** no. 13, **c** no. 14, **d** no. 15, and **e** an artificially matured eel. The zona radiata interna (ZRI) of the oocyte of no. 12 had eight layers (alternating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

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 reverse-transcription polymerase chain reaction (RT-PCR), mRNA expression of *zpb* and *zpc* in the European eel *Anguilla anguilla* was found to decrease from the mid-vitellogenic stage to the late-vitellogenic stage [53]. Considering these findings together, we suggest that formation of the chorion may cease after the mid-vitellogenic 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 seabream *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 change in a cyclic pattern, 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 oversynthesized; 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 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

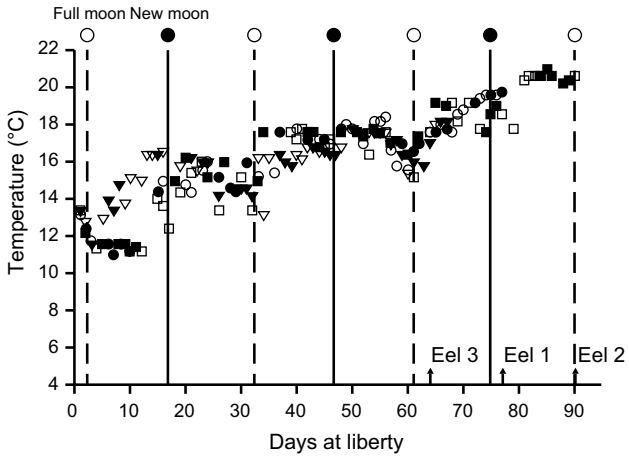

Fig. 6 Relationship between lunar cycle and nighttime water temperature in 3 individual New Zealand longfinned eels *Anguilla dieffenbachii* during their oceanic spawning migration (modified from Fig. 1 in Jellyman and Tsukamoto [60]). *Filled symbols*: average of a complete 12 h dataset; *unfilled symbols*: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

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.

Acknowledgments We thank Captain Nobuyuki Nagai and the crews of R/V Kaiyo Maru for their help with sample collection and Hideki Tanaka, Daisuke Ambe, Akira Shinoda, and Masanori Takahashi for onboard sampling assistance. We are also grateful to Noriaki Matsuya and the other members of the Laboratory of Fish Reproductive Physiology at the Graduate School of Fisheries Sciences, Hokkaido University, who helped with the experiments. We gratefully acknowledge the support of the Fishery Agency of Japan and the Fisheries Research Agency. This study was partly supported by JSPS KAKENHI grant no. 21228005, and the first author (H.I.) was supported by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science (no. 25–1576).

References

1. Tesch FW (1977) The eel: biology and management of anguillid eels. Chapman & Hall, London
2. Aoyama J, Miller MJ (2003) The silver eel. In: Aida K, Tsukamoto K, Yamauchi K (eds) Eel biology. Springer, Tokyo, pp 107–117
3. Haro A (2003) Downstream migration of silver-phase anguillid eels. In: Aida K, Tsukamoto K, Yamauchi K (eds) Eel biology. Springer, Tokyo, pp 215–222
4. Tanaka H, Kagawa H, Ohta H, Unuma T, Nomura K (2003) The first production of glass eel in captivity: fish reproductive physiology facilitates great progress in aquaculture. Fish Physiol Biochem 28:493–497
5. Tatsukawa K (2003) Eel resources in East Asia. In: Aida K, Tsukamoto K, Yamauchi K (eds) Eel biology. Springer, Tokyo, pp 293–298
6. Yamamoto K, Yamauchi K (1974) Sexual maturation of Japanese eel and production of eel larvae in the aquarium. Nature 251:220–222
7. Ohta H, Kagawa H, Tanaka H, Okuzawa K, Hirose K (1996) Changes in fertilization and hatching rates with time after ovulation induced by 17,20 beta-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica*. Aquaculture 139:291–301
8. Ohta H, Izawa T (1996) Diluent for cool storage of the Japanese eel (*Anguilla japonica*) spermatozoa. Aquaculture 142:107–118
9. Kagawa H, Tanaka H, Ohta H, Okuzawa K, Iinuma N (1997) Induced ovulation by injection of 17, 20β-dihydroxy-4-pregnen-3-one in the artificially matured Japanese eel, with special reference to ovulation time. Fish Sci 63:365–367
10. Ohta H, Kagawa H, Tanaka H, Okuzawa K, Iinuma N, Hirose K (1997) Artificial induction of maturation and fertilization in the Japanese eel, *Anguilla japonica*. Fish Physiol Biochem 17:163–169
11. Kagawa H, Iinuma N, Tanaka H, Ohta H, Okuzawa K (1998) Effects of rearing period in seawater on induced maturation in female Japanese eel *Anguilla japonica*. Fish Sci 64:77–82
12. Tanaka H (2011) Studies on artificial fry production of Japanese eel. Nippon Suisan Gakkaishi 77:345–351 (in Japanese)
13. Kagawa H, Tanaka H, Ohta H, Unuma T, Nomura K (2005) The first success of glass eel production in the world: basic biology on fish reproduction advances new applied technology in aquaculture. Fish Physiol Biochem 31:193–199
14. Ijiri S, Tsukamoto K, Chow S, Kurogi H, Adachi S, Tanaka H (2011) Controlled reproduction in the Japanese eel (*Anguilla japonica*), past and present. Aquaculture Europe 36:13–17
15. Tsukamoto K, Lee TW, Fricke H (2003) Spawning area of the Japanese eel. In: Aida K, Tsukamoto K, Yamauchi K (eds) Eel biology. Springer, Tokyo, pp 121–140
16. Utoh T, Mikawa N, Okamura A, Yamada Y, Tanaka S, Horie N, Akazawa A, Oka HP (2004) Ovarian morphology of the Japanese eel in Mikawa Bay. J Fish Biol 64:502–513
17. Sudo R, Suetake H, Suzuki Y, Utoh T, Tanaka S, Aoyama J, Tsukamoto K (2011) Dynamics of reproductive hormones during downstream migration in females of the Japanese eel, *Anguilla japonica*. Zool Sci 28:180–188
18. Todd PR (1981) Morphometric changes, gonad histology, and fecundity estimates in migrating New Zealand freshwater eels (*Anguilla* spp.). N Z J Mar Freshw Res 15:155–170
19. Hagihara S, Aoyama J, Limbong D, Tsukamoto K (2012) Morphological and physiological changes of female tropical eels, *Anguilla celebesensis* and *Anguilla marmorata*, in relation to downstream migration. J Fish Biol 81:408–426
20. Saito K, Lokman PM, Young G, Ozaki Y, Matsubara H, Okumura H, Kazeto Y, Yoshiura Y, Aida K, Adachi S, Yamauchi K (2003) Follicle-stimulating hormone b, luteinizing hormone b and glycoprotein hormone a subunit mRNA levels in artificially maturing Japanese eel *Anguilla japonica* and naturally maturing New Zealand longfinned eel *Anguilla dieffenbachii*. Fish Sci 69:146–153
21. Matsubara H (2003) Studies on mechanism of ovarian steroid hormone synthesis and the artificial control of oocyte growth in Japanese eel, *Anguilla japonica*. PhD dissertation, Hokkaido University of Fisheries Sciences, Hakodate
22. Lokman PM, Watari H, Okumura H, Kazeto Y, Young G, Adachi S, Yamauchi K (2000) Spontaneous and induced oogenesis: morphological differences between oocytes from New Zealand longfinned and Japanese eels. In: Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO (eds) Proceedings of the 6th International Symposium on the Reproductive Physiology of Fish, Bergen
23. Yamamoto M, Yamagami K (1975) Electron microscopic studies on choriolysis by the hatching enzyme of the teleost, *Oryzias latipes*. Dev Biol 43:313–321
24. Tesoriero JV (1977) Formation of the chorion (zona pellucida) in the teleost, *Oryzias latipes*: i. Morphology of early oogenesis. J Ultrastruct Res 59:282–291
25. Toshimori K, Yasuzumi F (1976) The morphology and the function of the oocyte chorion in the teleost *Plecoglossus altivelis*. J Electron Microsc (Tokyo) 25:210
26. Arukwe A, Knudsen FR, Goksøyr A (1997) Fish zona radiata (eggshell) protein: a sensitive biomarker for environmental estrogens. Environ Health Perspect 105:418–422
27. Arukwe A, Goksøyr A (2003) Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp Hepatol 2:4
28. Litscher ES, Wassarman PM (2007) Egg extracellular coat proteins: from fish to mammals. Histol Histopathol 22:337–347
29. Lubzens E, Young G, Bobe J, Cerda J (2010) Oogenesis in teleosts: how fish eggs are formed. Gen Comp Endocrinol 165:367–389
30. Wedemeyer GA (1996) Physiology of fish in intensive culture systems. Chapman and Hall, New York
31. Jaramillo R, Goicoechea O, Garrido O, Molinari E (2009) *Salmo salar*: ultrastructural morphology of chorion, from normal and with hatching problems ova. Arch Med Vet 41:67–71 (in Spanish with English abstract)
32. Jaramillo R, Goicoechea O, Garrido O, Molinari E (2012) Electrophoretic characterization for both normal and hard chorion proteins of *Salmo salar*. Arch Med Vet 44:59–65 (in Spanish with English abstract)
33. Ohta H, Takano K, Izawa T, Yamauchi K (1983) Ultrastructure of the chorion and the micropyle of the Japanese eel *Anguilla japonica*. Nippon Suisan Gakkaishi 49:501
34. Kayaba T, Takeda N, Adachi S, Yamauchi K (2001) Ultrastructure of the oocytes of the Japanese eel *Anguilla japonica* during artificially induced sexual maturation. Fish Sci 67:870–879

35. Adachi S, Ijiri S, Kazeto Y, Yamauchi Y (2003) Oogenesis in the Japanese eel, *Anguilla japonica*. In: Aida K, Tsukamoto K, Yamauchi K (eds) Eel biology. Springer, Tokyo, pp 301–317
36. Tyler CR, Sumpter JP (1996) Oocyte growth and development in teleosts. Rev Fish Biol Fish 6:287–318
37. Hyllner SJ, Haux C (1992) Immunochemical detection of the major vitelline envelope proteins in the plasma and oocytes of the maturing female rainbow trout, *Oncorhynchus mykiss*. J Endocrinol 135:303–309
38. Murata K, Sugiyama H, Yasumasu S, Iuchi I, Yasumasu I, Yamagami K (1997) Cloning of cDNA and estrogen-induced hepatic gene expression for choriogenin H, a precursor protein of the fish egg envelope (chorion). Proc Natl Acad Sci USA 94:2050–2055
39. Shimizu M, Fukada H, Fujita T, Hiramatsu N, Hara A (2000) Serum levels of precursors to vitelline envelope proteins (choriogenins) in Sakhalin taimen after treatment with oestrogen and during oocyte growth. J Fish Biol 57:170–181
40. Fujita T, Fukada H, Shimizu M, Hiramatsu N, Hara A (2004) Quantification of serum levels of precursors to vitelline envelope proteins (choriogenins) and vitellogenin in estrogen treated masu salmon, *Oncorhynchus masou*. Gen Comp Endocrinol 136:49–57
41. Modig C, Modesto T, Canario A, Cerdà J, von Hofsten J, Olsson PE (2006) Molecular characterization and expression pattern of zona pellucida proteins in gilthead seabream (*Sparus aurata*). Biol Reprod 75:717–725
42. Matsubara H, Kazeto Y, Ijiri S, Lokman PM, Abe T, Seki S, Inaba K, Hirai T, Adachi S, Yamauchi K (2001) Changes in the expression of steroidogenic enzyme genes in the Japanese eel ovary during artificial maturation. In: Tsukamoto K, Ymauchi K (eds) Aida K. Proceedings of the International Symposium on Advances in Eel Biology, Tokyo, pp 213–215
43. Chow S, Kurogi H, Mochioka N, Kaji S, Okazaki M, Tsukamoto K (2009) Discovery of mature freshwater eels in the open ocean. Fish Sci 75:257–259
44. Kurogi H, Okazaki M, Mochioka N, Jinbo T, Hashimoto H, Takahashi M, Tawa A, Aoyama J, Shinoda A, Tsukamoto K, Tanaka H, Gen K, Kazeto Y, Chow S (2011) First capture of post-spawning female of the Japanese eel *Anguilla japonica* at the southern West Mariana Ridge. Fish Sci 77:199–205
45. Tsukamoto K, Chow S, Otake T, Kurogi H, Mochioka N, Miller MJ, Aoyama J, Kimura S, Watanabe S, Yoshinaga T, Shinoda A, Kuroki M, Oya M, Watanabe T, Hata K, Ijiri S, Kazeto Y, Nomura K, Tanaka H (2011) Oceanic spawning ecology of freshwater eels in the western North Pacific. Nat Commun 2:179
46. Chai Y, Tosaka R, Abe T, Sago K, Sago Y, Hatanaka E, Ijiri S, Adachi S (2010) The relationship between the developmental stage of oocytes in various seasons and the quality of the egg obtained by artificial maturation in the feminized Japanese eel *Anguilla japonica*. Suisan Zoshoku 58:269–278
47. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675
48. Utoh T, Horie N, Okamura A, Yamada Y, Tanaka S, Mikawa N, Akazawa A, Oka HP (2003) Oogenesis in the common Japanese conger, *Conger myriaster*. Fish Sci 69:181–188
49. Ma XX, Zhu JQ, Zhou H, Yang WX (2012) The formation of zona radiata in *Pseudosciaena crocea* revealed by light and transmission electron microscopy. Micron 43:435–444
50. Oka HP (1982) Studies on the artificial maturation in the Japanese eel, *Anguilla japonica*-IV. Dimensional changes in the characteristics of oocytes during the course of maturation (in Japanese with English abstract). Bull Shizuoka Pref Fish Exp Stn 16:51–66
51. Kudo N, Miura T, Miura C, Yamauchi K (2000) Expression and localization of eel testicular ZP-homologues in female Japanese eels (*Anguilla japonica*). Zool Sci 17:1297–1302
52. Sano K, Kawaguchi M, Yoshikawa M, Iuchi I, Yasumasu S (2010) Evolution of the teleostean zona pellucida gene inferred from the egg envelope protein genes of the Japanese eel, *Anguilla japonica*. FEBS J 277:4674–4684
53. Mazzeo I, Peñaranda DS, Gallego V, Hildahl J, Nourizadeh-Lillabadi R, Asturiano JF, Pérez L, Weltzien FA (2012) Variations in the gene expression of zona pellucida proteins, zpb and zpc, in female European eel (*Anguilla anguilla*) during induced sexual maturation. Gen Comp Endocrinol 178:338–346
54. Hiramatsu N, Shimizu M, Fukada H, Kitamura M, Ura K, Fuda H, Hara A (1997) Transition of serum vitellogenin cycle in Sakhalin taimen (*Hucho perryi*). Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 118:149–157
55. Fujita T, Fukada H, Shimizu M, Hiramatsu N, Hara A (2005) Annual changes in serum levels of two choriogenins and vitellogenin in masu salmon, *Oncorhynchus masou*. Comp Biochem Physiol B Biochem Mol Biol 141:211–217
56. Ravaglia MA, Maggese MC (2003) Ovarian follicle ultrastructure in the teleost *Synbranchus marmoratus* (Bloch, 1795), with special reference to the vitelline envelope development. Tissue Cell 35:9–17
57. Abascal FJ, Medina A (2005) Ultrastructure of oogenesis in the bluefin tuna, *Thunnus thynnus*. J Morphol 264:149–160
58. Modig C, Raldúa D, Cerdà J, Olsson PE (2008) Analysis of vitelline envelope synthesis and composition during early oocyte development in gilthead seabream (*Sparus aurata*). Mol Reprod Dev 75:1351–1360
59. SchabetsbergerR Okland F, Aarestrup K, Kalfatak D, Sichrowsky U, Tambets M, Dall'Olmo G, Kaiser R, Miller PI (2013) Oceanic migration behaviour of tropical Pacific eels from Vanuatu. Mar Ecol Prog Ser 475:177–190
60. Jellyman D, Tsukamoto K (2010) Vertical migrations may control maturation in migrating female *Anguilla dieffenbachii*. Mar Ecol Prog Ser 404:241–247
61. Sato N, Kawazoe I, Suzuki Y, Aida K (2006) Effects of temperature on vitellogenesis in Japanese eel *Anguilla japonica*. Fish Sci 7:961–966
62. Sudo R, Tosaka R, Ijiri S, Adachi S, Suetake H, Suzuki Y, Horie N, Tanaka S, Aoyama J, Tsukamoto K (2011) Effect of temperature decrease on oocyte development, sex steroids, and gonadotropin beta-subunit mRNA expression levels in female Japanese eel *Anguilla japonica*. Fish Sci 77:575–582
63. Pérez L, Peñaranda DS, Dufour S, Baloche S, Palstra AP, Van Den Thillart GEEJM, Asturiano JF (2011) Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. Gen Comp Endocrinol 1174:51–59