1 **Title:** Development of oocytes in a post-spawning Japanese eel *Anguilla japonica*
 2 Temminck & Schlegel in captivity

3 **Running title:** Developing oocytes in a post-spawning eel

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17 **Abstract**

18 It is generally believed that parent freshwater eels (*Anguilla* sp.) die soon after spawning
19 on the assumption that eels are a semelparous (or monocyclic) fish (spawn once at the
20 last stage of life) like Pacific salmonids. However, we observed for the first time a
21 post-spawning female *Anguilla japonica* again possessed developing oocytes reaching
22 the final maturation stage in captivity five months after the last spawning even without
23 hormonal treatment. Here we describe information on this female about its biological
24 characteristics including gonadal histology and endocrine profiles. The data suggest that
25 lowering water temperature for a period of time is one of the important factors
26 influencing spontaneous gonadal development in this specimen. We also discuss the
27 possibility of induced multiple spawning of this species in captivity.

28 **Key words:** semelparity, anguillid eel, artificial maturation, spontaneous maturation,
29 water temperature

30 Freshwater eels (*Anguilla* sp.) are a food resource of worldwide importance, but they
 31 are becoming globally endangered, especially in three temperate species European eel
 32 *Anguilla anguilla* L., American eel *A. rostrata* (Lesueur, 1817) and *A. japonica*
 33 Temminck & Schlegel (Casselman 2003; Dekker 2009; Tsukamoto, Aoyama & Miller
 34 2009). To conserve these eels, techniques for artificial maturation of parent eels by
 35 hormonal administration and production of glass eels as seedlings for aquaculture
 36 industries have been intensively studied over the past four decades (Yamamoto &
 37 Yamauchi 1974; Tanaka 2003; Pedersen 2003; Oliveira & Hable 2010).

38 Usually, artificially matured parent eels by hormonal treatments are ordinarily
 39 discarded after spawning on the assumption that eels are a semelparous (or
 40 monocyclic) fish (spawn once at the last stage of life) like Pacific salmonids
 41 (*Oncorhynchus* sp.) (Wallace & Selman 1981; Murua & Saborido-Rey 2003).
 42 However, we observed for the first time a post-spawning female *A. japonica* again
 43 possessed developing oocytes reaching the final maturation stage in captivity five
 44 months after the last spawning even without hormonal treatment. Here we describe
 45 information on this female about its biological characteristics including gonadal
 46 histology and endocrine profiles, and we discuss the possibility of induced multiple
 47 spawning of this species in captivity.

48 A female silver eel of 67 cm in total length (TL) and 445 g in body weight
 49 (BW) was captured by a set net in Mikawa Bay (34°N, 137°E) on 19 November 1996
 50 (day 0). Estimated age by the number of the otolith annuli was eight. After
 51 acclimatization to an indoor tank (10,000 l) of sea water (34.5 practical salinity unit
 52 (PSU)) maintained at 18-20 °C for 2 weeks (day 13), the female was artificially
 53 induced to maturation by repeated injection of salmon pituitary extract (SPE, 20 mg kg
 54 BW⁻¹, once a week) for 6 weeks (Ohta, Kagawa, Tanaka, Okuzawa, Iinuma & Hirose

1997; Kagawa 2003) (Fig. 1). Some males (200–300 g BW), which were purchased from a commercial dealer, were also injected repeatedly with human chorionic gonadotropin (Sankyo, Tokyo, Japan) at a dose of 200 IU per fish once a week at 20 °C until full maturation (Ohta et al. 1997). Then the female was finally injected with luteinizing hormone-releasing hormone (LH-RH, 1 mg kg BW⁻¹) (Satoh, Yamamori & Hibiya 1992), and set in the 10,000-L tank together with some matured males (day 58). Two days later (day 60), the female spontaneously released approximately 140,000 eggs based on calculation by the subtraction of BW after spawning (406 g) from that before spawning (493 g). Fertilization rate were about 38.9% and the diameters of fertilized eggs was 1.55–1.65 mm, similar to *A. japonica* fertilized eggs collected at the southern part of the Mariana Ridge (1.49–1.71 mm; n = 12) (Yoshinaga, Miller, Yokouchi, Otake, Kimura, Aoyama, Watanabe, Shinoda, Oya, Miyazaki, Zenimoto, Sudo, Takahashi, Ahn, Manabe, Hagihara, Morioka, Itakura, Machida, Ban, Shiozaki, Ai & Tsukamoto 2011). About 40,000 larvae hatched and survived until 10 days after hatching without feeding. The number of eggs released by the female was considerably smaller than the estimated fecundity of this species ranged from 5 to 10 million eggs for female silver eels (35.7 to 92.4 cm TL) captured at Mikawa Bay (Matsui 1972), suggesting that almost all oocytes possibly remained to be spawned in the abdomen. However, the abdomen gradually shrunk, and the genital pore was completely closed soon after spawning.

Two days after spawning (day 62), the female was moved to an outdoor tank (2,000 l) of seawater (31.5 PSU) and kept for 134 days without diets (Fig. 1). The water temperature of the tank was once decreased to 10.7°C on day 122 and increased gradually to 14.3 °C until day 196. On day 196, we found that the body of the specimen was markedly thinned compared to the initial body, but the abdomen was

80 somewhat swelling and pliable despite the long-term starvation. Therefore, in
81 anticipation of second spawning, the female was again moved to the indoor tank of
82 water temperature at 22°C and kept for 22 days (Fig. 1). However, spawning did not
83 occur, so we dissected the female after heavy anesthetization on day 218 to observe the
84 gonads macroscopically and microscopically according to the procedure described in
85 Yamamoto, Omori & Yamauchi (1974). The BW of the specimen was decreased to
86 245 g, about 60% of the BW just after spawning, on day 218 (Fig. 1). Although we
87 have no detailed data on during the starvation, there is the possibility that the female
88 once attained a weight of less than 245 g and the weight increased again as the result of
89 oocyte hydration.

90 The female had apparently advanced ovaries with gonad-somatic index (GSI =
91 100 GW BW^{-1} ; GW: gonad weight) value of 15.4 compared with those of migrating
92 female eels caught at the coastal waters of Mikawa Bay (range: 0.2-4.3, n=286) (Utoh,
93 Mikawa, Okamura, Yamada, Akazawa, Tanaka, Horie & Oka 2004), whereas their GSI
94 values can reach 17.8-51.4 (n=20) at their spawning by artificial maturation (Okamura,
95 Yamada, Horie, Utoh, Mikawa, Tanaka & Tsukamoto 2008). Part of this high GSI
96 value is probably due to a loss of body weight, however, the thickness of ovaries was
97 enlarged compared with those found in migrating females captured at coastal waters
98 (Utoh *et al.* 2004) and its colour was yellow/white (Fig. 2a) and contained healthy
99 oocytes (*ca.* 700 μm in mean diameter). Histology of the ovaries showed that each
100 oocyte included a migrating germinal vesicle but there were no large lipid droplets
101 visible (Fig. 2b), corresponding to those of the early migratory nucleus stage with
102 oocyte diameters of 600-700 μm found in artificially matured Japanese eels
103 (Yamamoto *et al.* 1974). However, the number and size of yolk globule in these
104 oocytes seemed to be somewhat smaller (Fig. 2b), compared with those often found in

105 artificially matured eels at the same stage (Adachi, Ijiri, Kazeto & Yamauchi 2003).
 106 Some of degenerating oocytes were also observed (Fig. 2b). Furthermore, the GSI
 107 value of this female is similar to those of post-spawning female Japanese eels caught at
 108 the spawning area in the western North Pacific (9.0-13.4, n=3) (Tsukamoto, Chow,
 109 Otake, Kurogi, Mochioka, Miller, Aoyama, Kimura, Watanabe, Yoshinaga, Shinoda,
 110 Kuroki, Oya, Watanabe, Hata, Ijiri, Kazeto, Nomura, & Tanaka 2011). These
 111 post-spawning eels had also relatively advanced ovaries including oocytes at the
 112 mid-vitellogenic stage with oocyte diameters of 350-600 μm .

113 Estradiol-17 β (E2) and vitellogenin levels in the serum were also measured by
 114 the methods described elsewhere (Asahina, Kambegawa & Higashi 1995; Mikawa,
 115 Utoh & Oka, 2001; Utoh, Horie, Mikawa, Okamura, Yamada, Akazawa, Tanaka & Oka
 116 2005). Serum E₂ levels detected in this female (0.069 ng ml⁻¹) was apparently lower
 117 than those usually found in artificially matured eels at the migratory nucleus stages
 118 (range: 9.4-12.3 ng ml⁻¹, n=75) (Matsubara, Lokman, Kazeto, Adachi & Yamauchi
 119 2005). This low E₂ levels, however, may be not strange at the post vitellogenic
 120 stage, because higher levels of E₂ often detected in artificially matured eels at the final
 121 maturation stage are considered to be a peculiar feature induced by SPE injections
 122 (Chiba, Iwatsuki, Hayami, Hara & Yamauchi 1994; Ijiri, Kazeto, Takeda, Chiba,
 123 Adachi & Yamauchi 1995). Generally, serum E₂ levels in other teleosts increase in
 124 parallel with the progress of vitellogenesis and the decrease at the final maturation
 125 stage (Fostier; Jalabert; Billard; Breton & Zohar 1983), suggesting that low E₂ levels
 126 found in the present female may reflect a normal endocrine profile which appears in
 127 wild eels under natural conditions.

128 Meanwhile, vitellogenin, which is the precursor of yolk protein synthesized in
 129 liver in response to E₂, was detected at 2.52 mg ml⁻¹ in the serum, which was a level

130 included in the range found in wild silver eels at the early- to mid-vitellogenic stages
 131 caught in Mikawa Bay (range: 0.1-6.5 mg ml⁻¹, n=144) (Mikawa *et al.* 2001), but was
 132 apparently lower than those found in artificially matured female silver eels (2.5-10 mg
 133 ml⁻¹ n=20) (Sato, Kawazoe, Suzuki & Aida 2000). This levels of vitellogenin found
 134 in the present female are possibly the reminder of circulating vitellogenin, because it
 135 should be no longer necessary at this stage.

136 These reproductive characteristics suggest that this female had been ready to
 137 undergo the final maturation phase. Then, why did the female possess developing
 138 oocytes five months after the previous spawning? One possibility is that developed
 139 oocytes were retained in the female's abdomen just as it was for five months. This
 140 possibility is suggested by the calculation that the number of eggs spawned by the
 141 female (140, 000 eggs) was considerably smaller than the usual fecundity of this
 142 species (5 to 10 million eggs). However, Changchun, Yujun, Zhengfeng, Yiqiang &
 143 Yejin (1980) observed that remnant oocytes in post-spawning Japanese eels were
 144 usually absorbed by at least two months after spawning in captivity. Therefore, we
 145 think that this possibility is low. Another possibility is that these oocytes newly
 146 developed after the previous spawning. The external color of ovaries in the female on
 147 day 218 (Fig. 1a) appeared to be fresh and healthy, which were considerably differed
 148 from those with a lot of ecchymoses usually found in females just after spawning in
 149 captivity (Mikawa, unpublished data), suggesting the de novo development of oocytes
 150 in this female. Because the serum levels of the salmon gonadotropin (GTH) injected
 151 into eels rapidly decrease to considerable low levels by four days and undetectable
 152 levels by 24 days after the last injection (Sato, Kawazoe, Suzuki & Aida 1996; 2000),
 153 the influence of residual activity of SPE might be no longer available soon after
 154 spawning. If so, we suppose that the development of these oocytes had been

155 controlled by endogenous hormones.

156 Generally, it has been known that eels do not sexually mature in captivity

157 without exogenous hormonal assistance, because their endogenous hormonal systems

158 do not work under captive conditions, probably due to a lack of appropriate

159 environmental triggers for maturation (Adachi *et al.* 2003; Palstra, Ginneken & van

160 den Thillart 2009). Perhaps, relatively lower water temperature for five months

161 experienced by the present female is likely one of important factors influencing its

162 gonadal development. Tracking experiment of silver *A. anguilla* revealed that they

163 undergo diel vertical migration during the spawning migration between 300 and 500 m

164 in depth where temperatures were lower than 13°C (Tesch 1989). Another tracking

165 study showed that silver *A. dieffenbachii* Gray occasionally dove to around 6°C

166 (Jellyman & Tsukamoto 2010). A laboratory experiment also indicated that a variable

167 thermal regime that increased from lower (10 °C) to higher temperatures (14 and

168 17 °C) induced a faster gonadal development, higher gonadotropin β -subunits (fsh β

169 and lh β) expression and higher serum E2 levels during vitellogenesis in European

170 female eels injected with carp pituitary (Pérez, Peñaranda, Dufour, Baloché, Palstra,

171 van den Thillart & Asturiano 2011). In a marine eel, *Conger myriaster* (Brevoort),

172 gonadal development completes without hormonal treatment in captivity at 6°C and

173 they spawn several days after an abrupt temperature elevation to 10 °C (Utoh & Horie

174 2011). Therefore, it is possible that relatively lower water temperatures (11-14°C) for

175 five months stimulated pituitary hormonal axis and induced spontaneous oocyte

176 development. The abrupt elevation of water temperature from 14 to 22°C (Fig. 1),

177 however, might be unfavorable for the female reaching final maturation phase. Satoh

178 *et al.* (1992) and Dou, Yamada, Okamura, Shinoda, Tanaka & Tsukamoto (2007)

179 showed that spontaneous spawning of artificial matured *A. japonica* is efficiently

180 induced by an elevation of water temperature from 18 or 20 to 22°C. However, the
181 present female did not spawn after the temperature elevation from 14 to 22°C. It is
182 possible that the timing or width of temperature elevation was not adequate for the
183 female in the present study. These inadequate temperature conditions during the final
184 maturation phase were likely reflected in a relatively small number of yolk globules in
185 oocytes and some of degenerating oocytes found in this female (Fig. 2b).

186 Previously, Changchun *et al.* (1980) also observed histologically a number of
187 newly developed oocytes at the pre-vitellogenic stage in post-spawning Japanese eels
188 (n=23) two years after spawning and they did not die but ingested diets and regained
189 their BW after spawning. Horie *et al.* (unpublished data) observed that a number of
190 post-spawning *A. japonica* females repeatedly spawned (at least 3 times) in captivity
191 two or three weeks following previous spawning events when they were continually
192 received hormonal administration. These evidences suggest that eel ovary is possibly
193 polycyclic and the type of ovarian development of organization in this species might
194 be ‘group-synchronous’ rather than ‘synchronous’ (Wallace & Selman 1981; Murua &
195 Saborido-Rey 2003).

196 In conclusion, the post-spawning female Japanese eel possessed almost
197 matured oocytes five months after spawning, suggesting that captive eels have the
198 potential of multiple spawning. In addition, lower water temperatures for a period of
199 time may be one of the important factors influencing spontaneous gonadal
200 development in this species, which may lead to establishing a technique to obtain
201 efficiently eggs or to induce naturally eel maturation without exogenous hormones.

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316

317 **Figure legends**

318 **Figure 1** Changes in body weight (dotted line) of the *Anguilla japonica* female,
319 which was once induced spawning and then reared for about 5 months, and water
320 temperature experienced by the female (solid line). Arrows indicate the timings of
321 hormonal injection.

322

323 **Figure 2** Macroscopic image of ovaries (a) and histological image of oocytes (b) in
324 the *Anguilla japonica* female reared for about 5 months after the last spawning. G,
325 gut; L, liver; O, ovary; S, swim bladder; U, urogenital pore; d, oocyte atresia; n
326 nucleus.

° C

FIG. 1.

FIG. 2.