Zoological Studies **60**:61 (2021)
doi:10.6620/ZS.2021.60-61

Zoological Studies

Open Access

Histological Evidence of Multiple Spawning in Wild Female Japanese Eel *Anguilla japonica*

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Received 13 May 2021 / Accepted 14 July 2021 / Published 1 October 2021
Communicated by Pung-pung Hwang

The post ovulatory follicle (POF) is an important and reliable tissue structure used to investigate the spawning history in teleost fish. Fresh POFs shortly after spawning are comprised of cellular (follicular cells) and acellular (basement membrane and fibrils such as elastic fibers) components. The cellular components are quickly disintegrated by means of apoptosis, while the acellular components persist for a longer period. Since cellular components are well visualized by conventional hematoxylin-eosin (HE) staining but acellular components are not stained well, old POFs that have lost cellular components are difficult to identify. In this study, periodic acid-Schiff and Victoria blue staining, which can distinctly visualize acellular POF components, were applied to the ovarian tissues of Japanese eel (*Anguilla japonica*) (*n* = 9) captured from June to August of 2008, 2009, and 2013 at the southern West Mariana Ridge, a spawning area for Japanese eels. Only new POFs were observed in seven females caught in June, and these females had ovaries with early- to mid-vitellogenic stage oocytes. Both fresh and old POFs were observed in a female caught in July, and only mid-vitellogenic stage oocytes were observed. Only old POFs and no vitellogenic stage oocyte were observed in a female caught in August. A progressive decrease in muscle lipid content, gonad somatic index, and condition factors was observed from June to August. Thus, the female Japanese eel can spawn at least twice or three times at most during spawning season, depending on energy reserve.

Key words: *Anguilla japonica*, Wild female, Histology, Ovary, Multiple spawning.

BACKGROUND

A major breakthrough in the reproductive biology of catadromous eels was achieved through a series of scientific research cruises of the research vessel R/V Kaiyo-Maru, which is operated by Fisheries Agency of Japan and the Japan Fisheries Research and Education Agency. During these cruises, matured males and post-

Citation: Shimizu A, Ijiri S, Izumi H, Gen K, Kurogi H, Hashimoto H, Tanaka H, Jinbo T, Saito H, Chow S. 2021. Histological evidence of multiple spawning in wild female Japanese eel *Anguilla japonica*. Zool Stud **60**:61. doi:10.6620/ZS.2021.60-61.

spawning females of Japanese eel (*Anguilla japonica*) and giant mottled eel (*A. marmorata*) were captured in 2008 for the first time (Chow et al. 2009; Kurogi et al. 2011), followed by the first collection of fertilized Japanese eel eggs in 2009 (Tsukamoto et al. 2011). Among several new insights obtained from the collected adults, larvae, and fertilized eggs (Chow et al. 2009 2010 2017 2019; Kurogi et al. 2011; Tsukamoto et al. 2011; Izumi et al. 2015; Saito et al. 2015; Igarashi et al. 2018; Otake et al. 2019), the most notable is that the female may be a polycyclic (multiple) spawner (Tsukamoto et al. 2011). Otolith microstructure analysis of leptocephali and glass eels indicated that Japanese eels spawn during the new moon (Tsukamoto 1990; Ishikawa et al. 2001; Tsukamoto et al. 2003); this has been corroborated by the collection of the fertilized eggs, the newly hatched leptocephali, and the ovulated female during the new moon period (Tsukamoto 2006; Kurogi et al. 2011; Tsukamoto et al. 2011 2013; Aoyama et al. 2014). It has been a long-standing dogma that catadromous eels like Pacific salmon die once they have spawned (semelparous) (Aoyama and Miller 2003), but nobody has ever witnessed wild eels spawning. Tsukamoto et al. (2011) histologically examined Japanese eels caught in June 2009 and observed that the ovaries from four females contained mid-vitellogenic oocytes and postovulatory follicles (POFs), suggesting that catadromous eels may be polycyclic spawner. Recently, Palstra et al. (2020) reported that a female European eel (*Anguilla anguilla*) matured spontaneously in captivity and observed that only half of the oocytes were hydrated and matured, thus suggesting the species to be polycyclic spawner.

The post ovulatory follicle (POF) is an important and reliable tissue structure used to investigate the spawning history in teleost fish. The ovarian follicle consists of an oocyte surrounded by two layers—granulosa and theca—with a basement membrane (membrana propria folliculi) in between. The granulosa layer is a simple epithelium consisted of granulosa cells. The theca layer is a kind of connective tissue containing theca cells and fibrillar layers (normally collagen fibers and elastic fibers). Shortly after spawning, the cellular components (follicular cells) and acellular components are stratified and folded into the follicular lumen (Lang 1981), which is determined to be fresh POF. The fresh POFs can be easily observed using conventional hematoxylin-eosin (HE) staining, which visualizes the cellular components well. The number of follicular cells decreases rapidly by means of apoptosis (Drummond et al. 2000), whereas the acellular components persist for a longer time (Lang 1981; Santos et al. 2005; Thomé et al. 2006; Witthames et al. 2010; García-Seoane et al. 2014). Since HE staining is much less able to visualize the acellular components (Okochi et al. 2016), old POFs that have lost follicular cells are likely to be overlooked. All POFs observed in Japanese eel by Tsukamoto et al. (2011) using HE staining are fresh POFs, and their presence is evidence that the females have spawned. However, the coexistence of fresh POFs and vitellogenic oocytes is not direct evidence of multiple spawning, as we cannot know whether the vitellogenic oocytes observed develop for the next spawning. Hence, the coexistence of old and fresh POFs is a prerequisite to prove whether multiple spawning has occurred.

Periodic acid-Schiff (PAS) and Victoria blue (VB) staining are useful for visualizing the basement membrane and elastic fibers, respectively. Using PAS staining, old POFs were identified in Atlantic cod (*Gadus morhua*), a cold-water fish, at least 104 days after spawning (Witthames et al. 2010). Ayu (*Plecoglossus altivelis*), a temperate amphidromous fish, had been believed to live one year and die after a single spawning event, except in the case of the land-locked dwarf type (Matsuyama and Matsuura 1982 1984), but Shimizu et al. (2007) using PAS staining identified three different POF types, confirming that the species can spawn several times in a reproductive season. In the Pacific bluefin tuna (*Thunnus orientalis*), daily spawning has been confirmed by the detection of individuals with both new and old POFs (Okochi et al. 2016). Recently, VB staining was used to visualize elastic fibers in the ovary of the Pacific saury (*Cololabis saira*), identifying yet unspawned fish and post-spawning fish (Suyama et al. 2016).

In June and July of 2013, we caught an additional four Japanese eel females on the R/V Kaiyo-Maru. In this study, we applied histochemical analyses to the Japanese eel females available to date and reported histochemical evidence of multiple spawning.

MATERIALS AND METHODS

Fish and tissue samples

A large mid-water trawl net was used aboard the R/V Kaiyo-Maru at the southern part of the West Mariana Ridge, as described in previous studies (Chow et al. 2009; Kurogi et al. 2011; Tsukamoto et al. 2011). Catch and biological information from 10 females are presented in table 1, which includes data on six females caught in 2008 and 2009 derived from Kurogi et al. (2011), Tsukamoto et al. (2011), and Saito et al. (2015). Of the six Japanese eel females caught during the 2008–2009 cruises (see Kurogi et al. 2011; Tsukamoto et al. 2011), two were caught on 31 of August, 2008 and four were caught on 23 and 25 of June, 2009. Of the four

female Japanese eels caught in 2013, three were caught on 11 of June and one on 9 of July. All individuals were caught during the new moon. Once caught, each eel's dorsal muscle (circa 1 g or more, depending on fish size) was dissected on board, and frozen at -60°C, then transferred to the laboratory of the Fisheries Resources Institute. All individuals were dissected on board to observe the gonads for sex identification. The whole ovaries of all the females, except the two caught in 2008, were dissected and weighed on board. Small pieces dissected from near the middle of the ovaries were fixed in Bouin solution, then kept for one day at 4°C before immersion in 70% ethanol. The ovaries were then kept at room temperature until examination. Since the ovaries from the two females (U8-1 and -2) caught in 2008 were vestigial (Kurogi et al. 2011), the whole ovary could not be dissected for weighing on board. The whole fish body was frozen at -60°C on board, then transferred to the laboratory. The ovarian tissue from one (U8-2) of these two females was not available for the present study. The whole body of the other female was fixed in 10% formalin for a week, then transferred to 70% ethanol. The ovarian piece of this individual was dissected for histological analysis in 2020.

Histological observation of the eel ovary

The ovarian tissue was dehydrated in an ethanol-butanol series, and embedded in Paraplast plus (Sigma-Aldrich, Saint Louis). Sections of 10 μm thickness were placed on glass slides. The specific stainings for the sections generally followed McManus (1948) for PAS and Sano and Mashimo (1965) for VB. The sections were de-paraffined with xylene and washed with an ethanol series, to which prestaining oxidizations were performed as followings: periodic acid (Wako, Osaka) treatment (0.5%, 5 min) for PAS staining or $KMnO_4$ (Wako, Osaka) treatment (0.15% with 0.15% sulfonic acid, 5 min), followed by de-coloration with 5% $NaHSO_3$ (Wako, Osaka) solution for VB staining. Then the sections were washed with water and stained with Schiff's solution (Wako, Osaka; for PAS) for 15 min or Victoria blue solution (Wako, Osaka; for VB) overnight. Counterstains were created using Mayer's hematoxylin (Wako, Osaka) for PAS, 0.2% azocarmin G solution (Wako, Osaka) for VB, or 0.8% Orange G solution (Wako, Osaka) for VB + PAS in the typical manner. For comparison, conventional HE staining was also performed in the typical manner.

Size distribution of follicles

Ovarian follicles were isolated with fine forceps from ovarian fragments that had been stored in 70% ethanol after Bouin's fixation, and a photograph was taken under a dissecting microscope. Since the thickness of the follicular cell layer covering the oocyte was negligible, follicle diameter was considered to be almost the same as the size of oocyte. The diameters of 100 randomly selected follicles were measured by Image J (NIH) software from digitally-photographed images under a light microscope. According to Kayaba et al. (2001) and Adachi et al. (2003), we determined size range of early-, mid- and late-vitellogenic stages to be 250 to 350, 400 to 500, and 600 to 700 μm, respectively.

Lipid content

The muscle pieces were weighed, immersed and homogenized in a reagent mixture containing

Table 1. Catch date and locality, total length (TL), body weight (BW), gonad weight (GW), gonadosomatic index (GSI), condition factor (CF), and % muscle lipid content of 10 females of *Anguilla japonica* caught at the southern part of the West Mariana Ridge

ID No.	Catch date	coordinate (N, E)	TL (mm)	BW (g)	GW (g)	GSI	CF	Lipid (%)
T7-1	2013/6/11	13.24°, 142.35°	560	118	11.4	9.7	0.670	3.0
T7-2	2013/6/11	13.24°, 142.35°	683	202	25.0	12.4	0.633	10.7
T7-3	2013/6/11	13.24°, 142.35°	616	128	17.3	13.5	0.547	3.0
AjF1[a]	2009/6/23	12.17°, 141.25°	749	244*	32.0*	13.1	0.581	12.1[c]
AjF2[a]	2009/6/23	12.17°, 141.25°	767	244	22.0	9.0	0.541	5.5[c]
AjF3[a]	2009/6/23	12.17°, 141.25°	739	330	37.0	11.2	0.817	15.2[c]
AjF4[a]	2009/6/25	12.17°, 141.25°	574	120	16.0	13.3	0.635	5.7[c]
T7-5	2013/7/9	12.53°, 141.37°	728	166	8.23	5.0	0.429	2.1
U8-1[a,b]	2008/8/31	14.07°, 142.44°	555	91	no data	no data	0.529	2.3[c]
U8-2[a,b]	2008/8/31	14.07°, 142.44°	662	117	no data	no data	0.403	1.4[c]

Reported by [a]Tsukamoto et al. (2011), [b]Kurogi et al. (2011), and [c]Saito et al. (2015). *Over-ripened ovulated eggs were not included. Ovarian tissue of U8-2 was not available.

chloroform and methanol (2:1, v/v). Total lipid of a homogenized sample was then extracted according to the procedure of Folch et al. (1957). Lipid content (%) was expressed as the weight of total lipid extracted per weight of muscle tissue used.

Statistical analysis

Non-parametric *t*-test (one-tailed) was used to compare gonadosomatic index (GSI), condition factor (CF), and % muscle lipid content between June and July plus August samples. Gonad weight could not be measured for two females in August, but we tentatively determined them to be 1 g. Since only one female in July and two in August were caught, data on these individuals were pooled to compare them with samples caught in June. Further, condition factor (CF) and % muscle lipid content data of seven female silver eels (total length: 440 to 764 mm) caught in 2008 at estuary and coastal areas of Japan were derived from Saito et al. (2015). Mann-Whitney *U*-test (one-tailed) was used to compare these values between silver female samples collected in Japan and spawning areas.

RESULTS

Biological characteristics of female eels

The lateral view and dissected body cavity of the two females (U8-1 and -2) caught in 2008 are given in Kurogi et al. (2011), and those from one (AjF1) of the four females in 2009 are presented in Tsukamoto et al. (2011). The lateral view and the external appearance of ovaries from the remaining three females (AjF2 to 4) in 2009 are shown in figure S1, and those of the four females (T7-1 to -3 and -5) caught in 2013 are shown in figure 1. The ovaries of the individuals caught in June were yellowish-white in color (Fig. 1b, d, f), while the ovary of an individual caught in July was reddish and somewhat smaller than those caught in June (Fig. 1h).

The averages of gonad somatic index (GSI), condition factor (CF), and % muscle lipid content for each month are presented in figure 2. Large variations in muscle lipid content were observed for the June individuals, but a progressive downward trend in all indices was observed from June to August. All indices from the June sample were significantly larger than those in the July + August samples ($p < 0.005$). Saito et al. (2015) reported muscle lipid contents (14.4 to 22.2 %) and CF (1.247 to 1.695) of female silver eels caught in Japan, and the averages of these indices were significantly larger than those of 10 females analyzed in the present study ($p < 0.005$) and of seven females caught in June ($p < 0.05$). The muscle lipid contents of the female silver eels caught in the spawning area ($n = 10$) (present study) and in Japan ($n = 7$) (Saito et al. 2015) were plotted against CF (Fig. 3), and a significantly positive correlation was observed in the former group ($p < 0.05$), but not in the latter group ($p = 0.48$).

Size distribution of follicles

Diameter distributions of follicles isolated from eight females caught in 2009 and 2013 are shown in figure 4. Follicle diameter distributions in seven females caught in June of 2009 and 2013 (Fig. 4a–g) were wider than that of a female caught in July 2013 (Fig. 4h). Size range of follicles indicated that the majority of oocytes of females in June were early- to mid-vitellogenic stage and those of a female in July were mid-vitellogenic stage. No small oocytes (smaller than 100 μm) were observed in any of the eight females examined.

Ovarian histology and histochemistry

Histological ovary sections from individuals caught in June, July, and August are shown in figure 5. Small oocytes such as perinucleolus stage ones were completely absent in all nine individuals examined in this study as well as one (U8-2) in August reported by Kurogi et al. (2011). Early- to mid-vitellogenic stage oocytes were abundant in females caught in June of 2009 and 2013 (Fig. 5a, d; also see Fig. S2a–g), while only mid-vitellogenic stage oocytes were observed in a female caught in July (Fig. 5b, e; also see Fig. S2h), corresponding to the follicle diameter distributions (Fig. 4). The ovary of two individuals caught in August of 2008 mainly consisted of connective tissue and blood vessels, and no oocyte was observed (Fig. 5c, f; also see Kurogi et al. 2011).

Histological ovary sections of females caught in June and July are shown in figure 6. POFs were abundant in the ovaries of these individuals (Fig. 6a, b; asterisks). Serial sections of a new POF with the enlarged views of a female caught in June are shown in figure 6c–h. PAS + VB staining (Fig. 6c, d), PAS staining (Fig. 6e, f), and VB staining (Fig. 6g, h) were applied to these sections, showing cellular components (originally follicle envelope cells) (Fig. 6c–h; daggers) and acellular components (prominently visualized PAS positive basement membrane and VB positive elastic fibers) (Fig. 6c–h; open arrows).

Histological ovary sections of females caught in July and August are shown in figure 7. In the ovary of an individual caught in July 2013, dense PAS and VB positive matrixes accompanying very few somatic cells

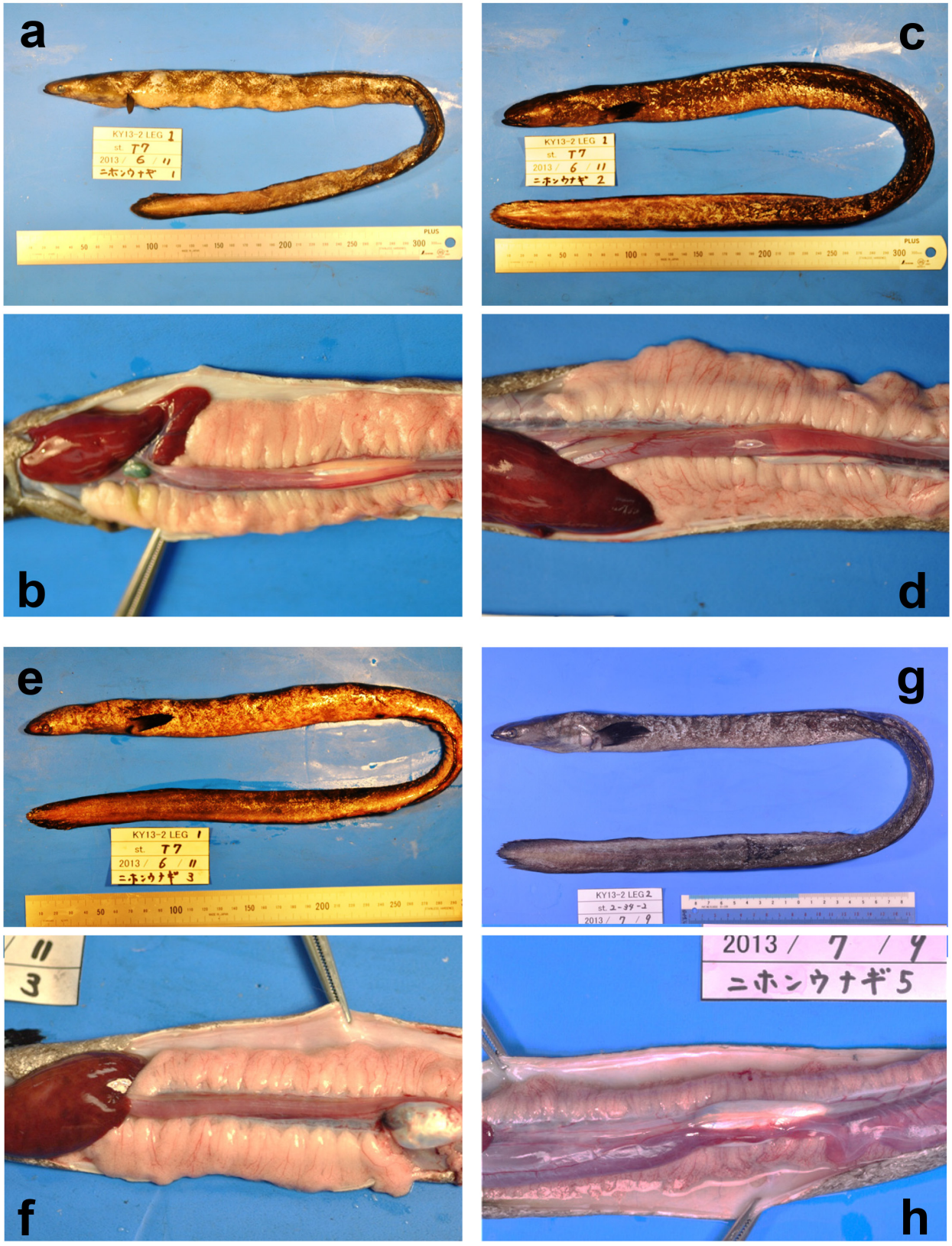

Fig. 1. Lateral views (a, c, e and g) of four females of Japanese eel (*Anguilla japonica*) caught at the West Mariana Ridge in 2013, and their ovarian appearance (b, d, f and h). a, b Specimen No. T7-1 caught on 11 of June. c, d Specimen No. T7-2 caught on 11 of June. e, f Specimen No. T7-3 caught on 11 of June. g, h Specimen No. T7-5 caught on 9 of July. See table 1 for individual information.

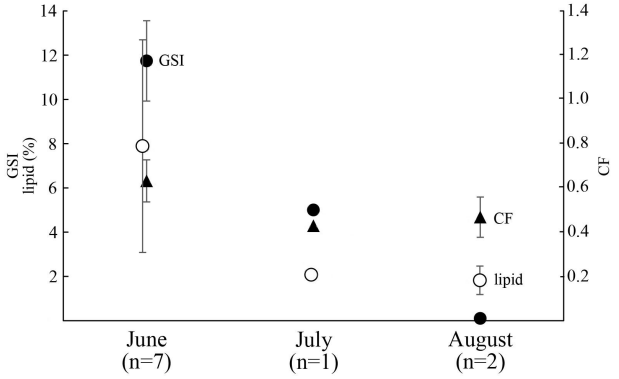

Fig. 2. Averages and standard deviations (vertical bar) of gonadosomatic index (GSI: closed circle), % muscle lipid content (lipid: open circle), and condition factor (CF: closed triangle) of 10 female silver eels caught in the spawning area.

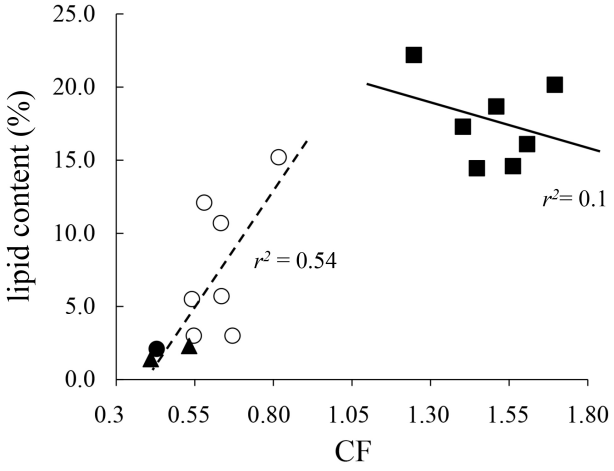

Fig. 3. Muscle lipid content (%) plotted against condition factor (CF) in 10 female silver eels of June (open circle), July (closed circle), and August (closed triangle) caught in the spawning area and seven silver females (closed square) caught in Japan.

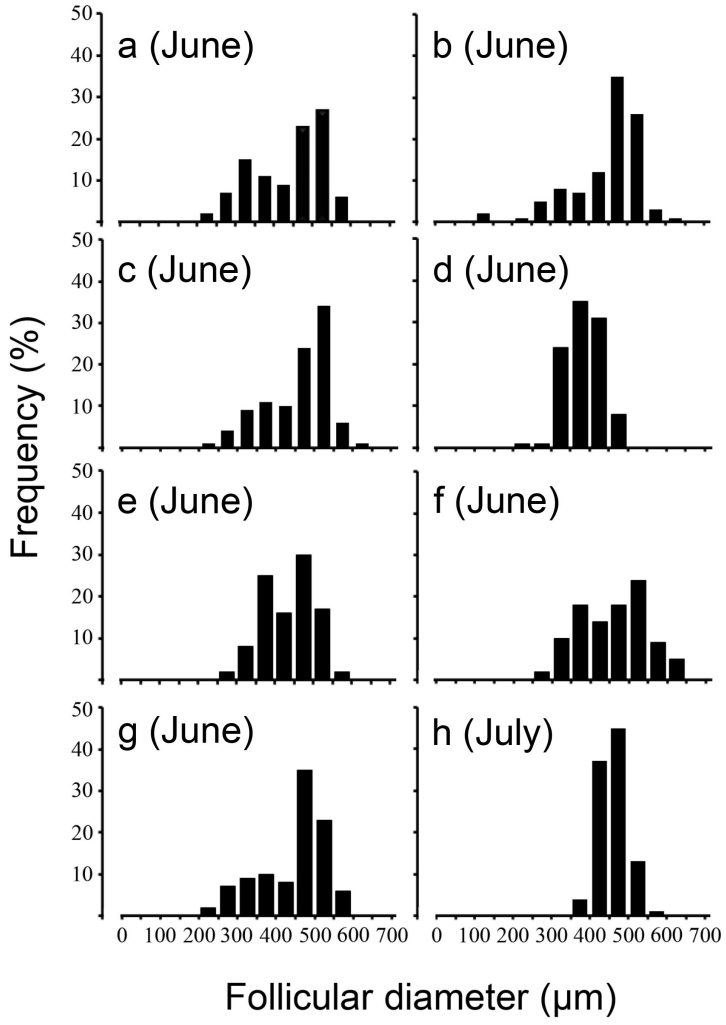

Fig. 4. Diameter distribution of follicles isolated from eight female Japanese eels caught in 2009 and 2013. a, T7-1. b, T7-2. c, T7-3. d, AjF1. e, AjF2. f, AjF3. g, AjF4. h, T7-5. See table 1 for individual information.

were observed (Fig. 7a, b). These were determined to be old POFs, because the acellular components were strongly visualized with PAS and VB staining and cellular components (S) were drastically reduced. New and old POFs were observed together (Fig. 7c, d), indicating that this female has spawned once in the previous month and again this month. The two POF types were clearly distinguished by the acellular components being fibrous in new POFs and lump-like in old ones, in addition to the scarceness of the cellular components in old POFs. In the ovary of a female (U8-1) caught in August 2008, old POFs were frequently observed, while new POFs were not present (Fig. 7e–g). The old POFs were roughly classified into two types, moderately condensed and highly condensed (Fig. 7h), although exact discrimination was difficult.

The composition of oocyte stages and the POF types observed in each female are summarized in table 2. Seven females caught in June had both early- and mid-vitellogenic stage oocytes, and only new POFs were observed in these females. Mid-vitellogenic stage oocytes but no early-vitellogenic stage oocytes were observed in a female caught in July. Both new and old POFs were observed in this female. Vitellogenic oocytes

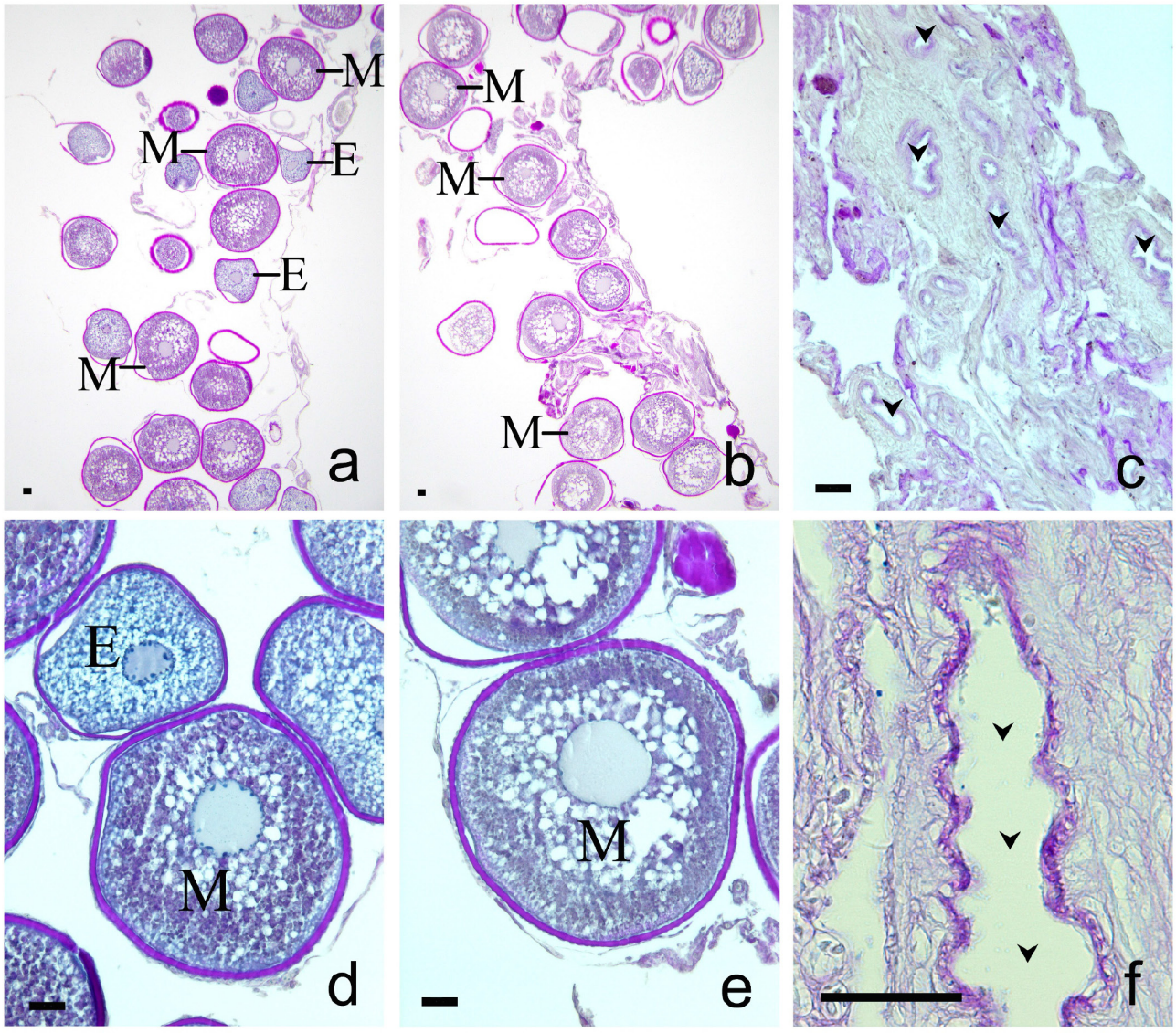

Fig. 5. Histological ovarian sections of females caught in June (a, d), July (b, e), and August (c, f). PAS staining with hematoxylin counterstain. Mid-vitellogenic stage oocytes (M) and early-vitellogenic stage oocytes (E) were observed in ovaries from females caught in June (a), whereas only mid-vitellogenic stage oocytes (M) were observed in the ovary of a female caught in July (b). Smaller oocytes (oocytes in perinucleolus or younger stages) are absent in these individuals caught in June and July (a, b). No oocytes but many blood vessels (arrowheads) were observed in the ovary of a female caught in August (c, f). Scale bar = 50 μm.

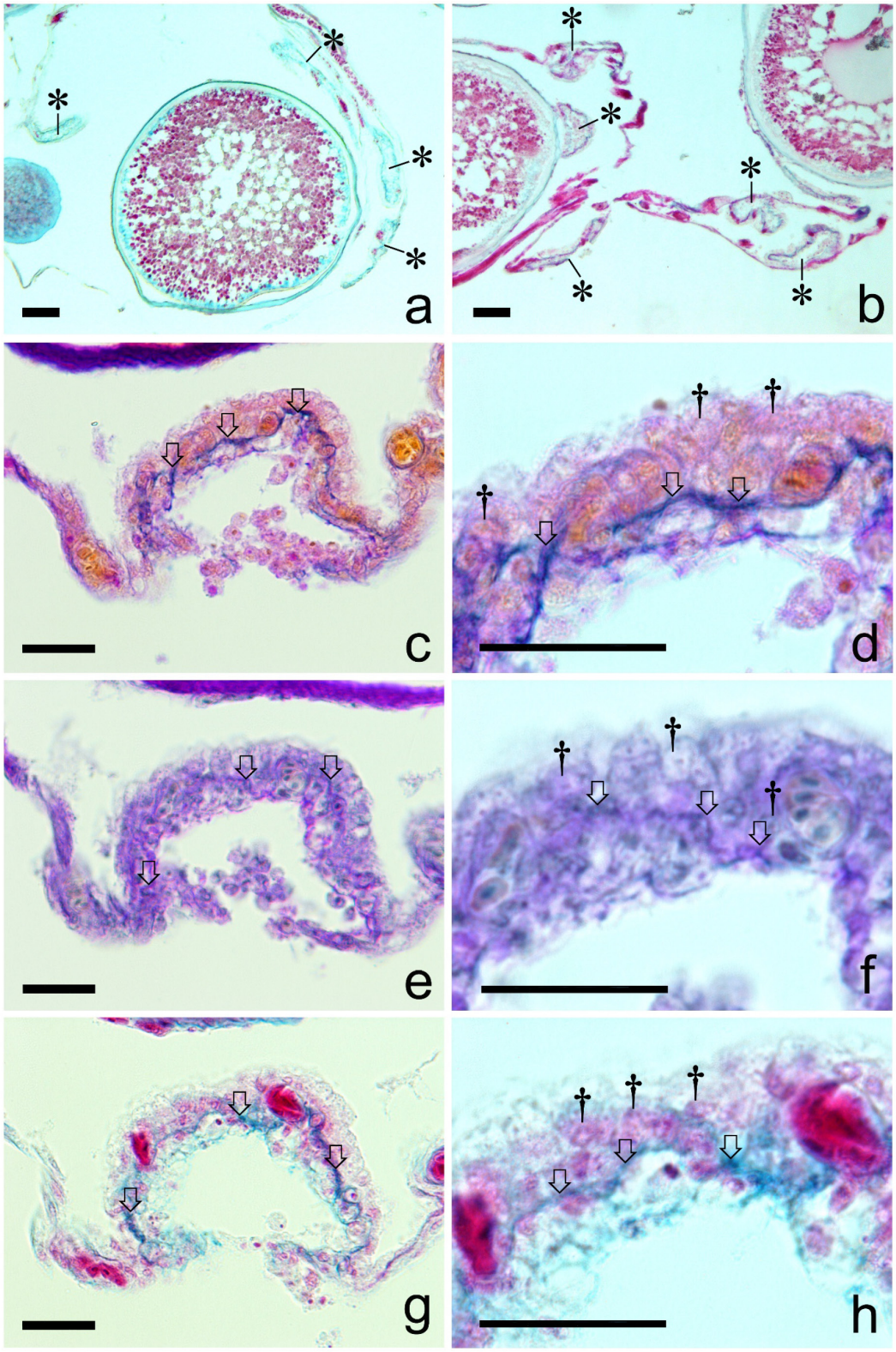

Fig. 6. Histological ovarian sections of females caught in June and July, showing post ovulatory follicles. a, b, Low magnification photomicrographs of ovarian sections of the females caught on 11 of June (a) and 9 July (b). VB staining. POFs (*) are abundantly seen. (c–h) Serial-sections (c, e and g) and the enlarged views (d, f and h) of new POFs in the ovary of the female caught on 11 of June. c, d, PAS+VB staining. e, f PAS staining. g, h VB staining. Daggers indicate cellular components (originally follicle envelope cells), and open arrows indicate the acellular components (basic membrane and/or elastic fibers). Scale bar = 30 µm.

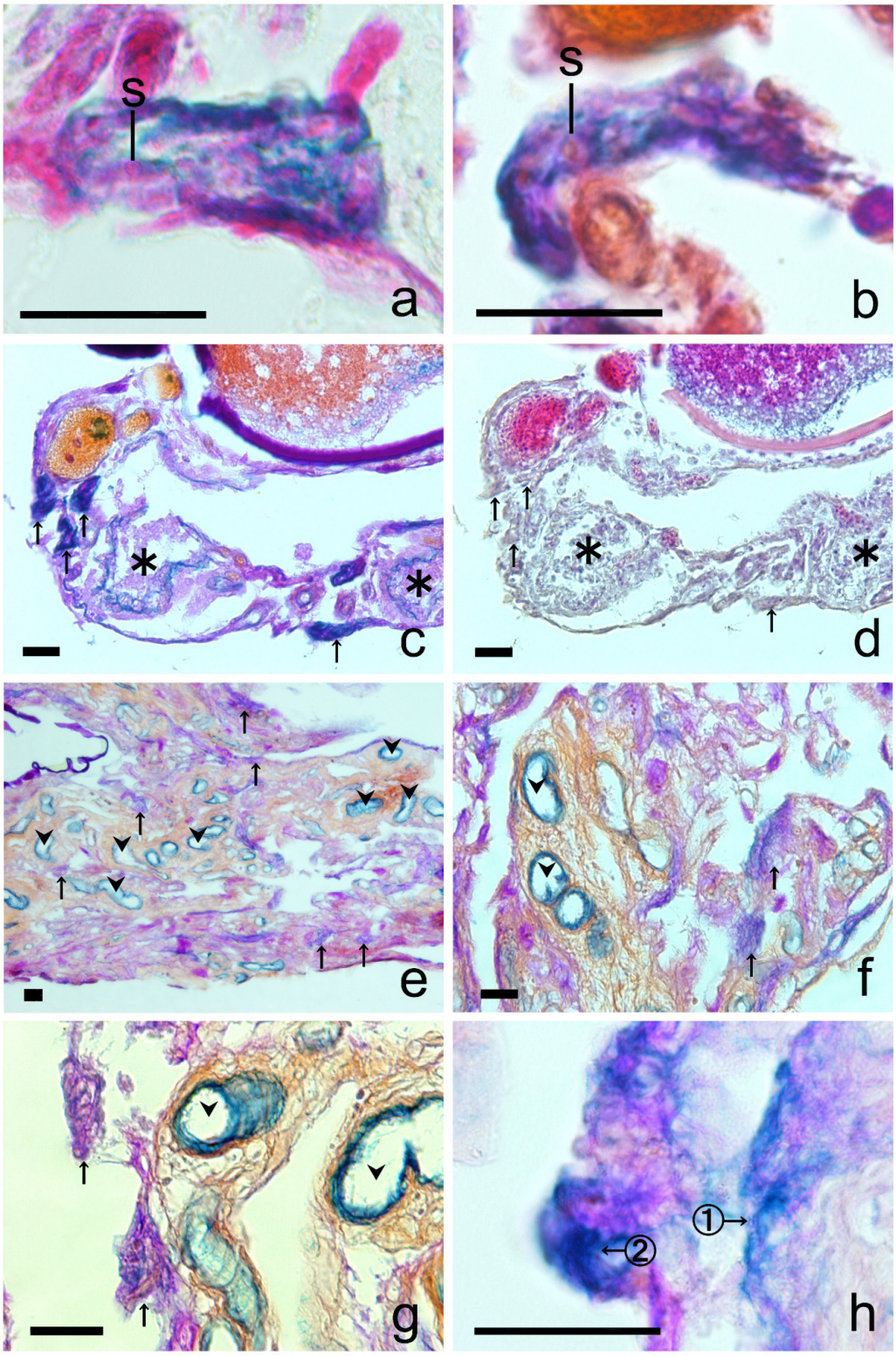

Fig. 7. Histological ovarian sections of females caught in July and August, showing post ovulatory follicles. a, b, Old POFs in the ovary of the female caught on 9 of July. a, VB staining. b, PAS + VB staining. Very few somatic cells (S) were observed. c, d Ovarian serial-sections of the female caught on 9 of July. c, PAS +VB staining. New (*) and old (arrows) POFs are identified. d Mayer's hematoxylin and eosin staining. Although new POFs (*) are identified, old POFs (arrows) are only faintly stained. e–h, The ovary of the female caught on 31 of August. PAS + VB staining. e–g Many old POFs (arrows) and blood vessels (arrowheads) are seen. h Old POFs are stained purple with both PAS and VB, in which moderately condensed types (1) and highly condensed types (2) were observed. Scale bar = 30 μm.

and new POFs were not observed in two females caught in August, and only old POFs were observed in a female analyzed in this study.

DISCUSSION

This study presents histological evidence of multiple spawning in wild female Japanese eel. Only new POFs were observed in seven females caught in June, indicating that these females experienced their first spawning during the new moon period of June. Mid- and early-vitellogenic stage oocytes observed in these females may be germ cell reserves for spawning in July and August, respectively. Ijiri et al. (1998) attempted artificial maturation of silver stage wild Japanese eel and observed that vitellogenic oocytes ranging from 500 to 600 μm took four to five weeks to reach their final maturation phase. This evidence supports our hypothesis that those wild eels after spawning could develop remaining mid-vitellogenic oocytes to the stage of oocyte maturation by the next new moon period. Characteristics of old POFs (condensation of the acellular components and reduction of the cellular component) determined in the present study were almost identical to those reported in other fish species (Murua et al. 2003; Shimizu et al. 2007; Okochi et al. 2016). Both new and old POFs were observed in a female caught in July and only mid-vitellogenic stage oocyte was observed in this female, indicating that this female spawned in June and July and was probably preparing for the last spawning in August. Vitellogenic oocytes and new POFs were not observed in two females caught in August (also see Kurogi et al. 2011), and only old POFs were observed in a female analyzed in this study, indicating that these females were no longer spawning and had finished their final spawning during the previous new moon period.

Fish spawning fecundity is largely categorized into determinate and indeterminate types (Hunter et al. 1992; Kurita and Kjesbu 2009; Lowerre-Barbieri et al. 2011; Murua and Saborido-Rey 2003; Ganias et al. 2015). Since small oocytes (less than 100 μm) are completely absent in all females, the Japanese eel must be the most strict determinate type spawner, in which the recruitment of new oocytes is terminated before the first spawning.

The histological characteristics of ovaries observed in June to August samples may strongly correlate with muscle lipid content, CF and GSI. Japanese eels perform long-distance oceanic migration from northeast Asia to their narrow spawning area in the West Mariana Ridge (Tsukamoto et al. 2003 2011). Even if many of the adult Japanese eels caught in the spawning area originated from Japan, as suggested by Otake et al. (2019), the departure area and the distance and time for eels to get to the spawning area may vary considerably. We determined that all females caught in June had just completed their first ovulation and spawning. Large variation in muscle lipid contents were observed among these females, which may correspond to variations in lipid contents at departure and the time and distance required for the oceanic migration. Wide variation in muscle lipid content among silver stage individuals caught in estuaries and coastal areas has been observed in the Japanese and European eels (Svedäng and Wickström 1997; Ozaki et al. 2008; Saito et al. 2015), which may also be the case for the females caught in June in the spawning area. Significantly lower muscle lipid contents, CF and GSI were observed in the females caught in July and August compared to those in June were attributable more likely to multiple spawning.

Table 2. Oocyte stages and types of POFs observed in 10 females

ID	catch date	vitellogenic oocyte stages		POFs	
				new	old
T7-1	2013/6/11	early[§]	mid	+	−
T7-2	2013/6/11	early[§]	mid[†]	+	−
T7-3	2013/6/11	early[§]	mid[†]	+	−
AjF1	2009/6/23	early[§]	mid	+	−
AjF2	2009/6/23	early	mid	+	−
AjF3	2009/6/23	early	mid[†]	+	−
AjF4	2009/6/25	early[§]	mid	+	−
T7-5	2013/7/9	−	mid	+	+
U8-1	2008/8/31	−	−	−	+
U8-2*	2008/8/31	−	−	−	no data

[§]including very small number of oocytes smaller than 250 μm. [†]including very small number of oocytes larger than 600 μm. *determined from histological sections by Kurogi et al. (2011). +: present, −: absent.

Declines in batch fecundity with spawning times have been reported in the ayu (Shimizu et al. 2007), which may also be the case for the Japanese eel in the spawning areas who showed progressive decreases in CF, GSI and lipid content. It was remarkable that no new POFs or vitellogenic oocytes were found in females caught in August (Kurogi et al. 2011; this study). These individuals had nearly spent in their energy and germ cell reserves but had survived for at least one month after the previous spawning. The number of possible spawning should depend on energy and germ cell reserves. Females that have exhausted almost all of their energy reserve by the time they arrived at the spawning area might end their life after a single spawning, whereas females with sufficient energy would be able to spawn again. Plankton surveys performed to date collected no preleptocephali and eggs in the spawning area before May, and very few were collected after September (Tsukamoto 2006; Kurogi et al. 2011; Tsukamoto et al. 2011 2013; Aoyama et al. 2014). Thus, female Japanese eels as a new moon spawner may have up to four chances to spawn from May to August. The June females determined to have just finished their first spawning possessed two batches of oocytes (early- and mid-vitellogenic stages) but no younger oocytes such as the perinucleolus stage. A female caught in July was determined to have spawned twice; this female had only mid-vitellogenic oocytes and considerably depressed muscle lipid content, CF and GSI. The females caught in August also had depressed muscle lipid content, CF and GSI; no oocyte was found in these females. These strongly suggest that female Japanese eels may spawn at most three times.

CONCLUSIONS

Catadromous eels of the genus *Anguilla* perform long distance spawning migration. It was believed that they die once they have spawned, but there is no information to support it. Tsukamoto et al. (2011) observed vitellogenic oocytes and postovulatory follicles (POFs) in female Japanese eel (*Anguilla japonica*) caught in the spawning area. Palstra et al. (2020) reported that a female European eel (*A. anguilla*) matured spontaneously in captivity and observed that only half of the oocytes were hydrated and matured. They suggested that catadromous eels may be a polycyclic spawner, but these histological observations also cannot be solid evidence. Fresh POFs comprising cellular (follicular cells) and acellular (basement membrane and fibrils such as elastic fibers) components can be seen in the ovaries shortly after spawning. The cellular components are quickly disintegrated by means of apoptosis, while the acellular components persist for a longer period, and this state is defined as an old POF. Coexistence of fresh and old POFs can be direct evidence for multiple spawning. Since cellular components are well visualized by conventional hematoxylin-eosin (HE) staining, all POFs observed by Tsukamoto et al. (2011) were fresh. Old POFs are more likely to be overlooked, as acellular components are not visualized well by HE staining. In this study, periodic acid-Schiff and Victoria blue staining that can distinctly visualize acellular POF components were applied to the ovarian tissues of Japanese eels captured in the spawning area. Only new POFs were observed in all seven females caught in July, and these females had early- to mid-vitellogenic stage oocytes in their ovary. Both fresh and old POFs were observed in a female caught in July, and only mid- vitellogenic stage oocytes were observed. A female caught in August had only old POFs and no oocyte. A progressive decrease in muscle lipid content, gonad somatic index, and condition factors was observed from June to August. These indicate that the female Japanese eel can spawn at least twice or three times at most during spawning season, depending on energy reserve.

Acknowledgments: We thank the captains and crew members of R/V Kaiyo-Maru from the Fisheries Agency of Japan for their invaluable support in the research cruise. We also thank A. Tawa, K. Nomura, T. Tomoda, S. Terashima, M. Okazaki, T. Watanabe, Y. Kazeto, Japan Fisheries Research and Education Agency, K. Tsukamoto, J. Aoyama, University of Tokyo, S. Watanabe, Kindai University, N. Mochioka, Kyushu University, for their invaluable contribution in the research cruise. We would also like to thank the three anonymous reviewers for their invaluable and constructive comments on this manuscript. This research has been financially supported by the Fisheries Agency of Japan and the Japan Fisheries Research and Education Agency.

Authors' contributions: Akio Shimizu and Seinen Chow conceived the ideas and designed the methodology. Shigeho Ijiri, Hiroaki Kurogi, Hashimoto Hiroshi, Hideki Tanaka, Tadao Jinbo, and Seinen Chow attended the research cruise. Akio Shimizu, Shigeho Ijiri, Hikari Izumi, Koichiro Gen, and Seinen Chow performed histological analyses. Hiroaki Saito and Seinen Chow performed lipid content analysis. Akio Shimizu and Seinen Chow wrote the first draft of the manuscript and all authors read, commented and approved the manuscript.

Competing interests: We declare that we have no

conflict of interest regarding this study.

Availability of data and materials: The datasets obtained in this study are available from the corresponding author upon reasonable request.

Consent for publication: All of the authors consent to the publication of this manuscript.

Ethics approval consent to participate: Not applicable.

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Supplementary materials

Fig. S1. Females of Japanese eel (*Anguilla japonica*) caught in June 2009. a, ID No. AjF2. b, ID No. AjF3. c, ID No. AjF4. Photographs of the individual of ID No. AjF1 are shown in Tsukamoto et al. (2011). See table 1 for individual information. (download)

Fig. S2. Low magnification photomicrographs of ovarian sections of the seven eels (a–g) captured in June and one (h) in July. PAS staining with hematoxiline counterstain. Scale bar = 100 μm. a, ID No. T7-1. b, ID No. T7-2. c, ID No. T7-3. d, ID No. AjF1. e, ID No. AjF2. f, ID No. AjF3. g, ID No. AjF4. h, ID No. T7-5. Note maldistribution of cellular components in some oocytes (*), which were considered to be postmortem changes as these fishes were left dead in trawl net for several hours. See table 1 for individual information. (download)