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Gonadal sex differentiation and development during early ontogenesis in the breeding kisslip cuttlefish (*Sepia lycidas*)

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ABSTRACT

To understand and obtain basic information on sex differentiation in the kisslip cuttlefish (*Sepia lycidas*), the gonadal sex differentiation process was investigated histologically. An undifferentiated gonad consisting of germ cells and somatic cells was found to form at a caudal site in the space between the internal yolk sacks of cuttlefish embryos at 14 and 21 days after spawning (DAS). Sexual dimorphism in the gonad was first detected at around 28 DAS. Meiotic oocytes were observed as the first visible morphological characteristic of ovaries in the gonads of some cuttlefish embryos at 28 DAS. In other individuals, neither meiotic germ cells, nor the appearance of a testicular structure, were observed in the gonad even after 10 days post hatching (DPH). Seminiferous tubules, consisting of a small number of spermatogonia and a surrounding basement membrane, were the first visible morphological characteristic of the testis in the male gonad, detected at around 20 DPH. This is the third report on the gonadal sex differentiation process in cephalopods.

1. Introduction

Cephalopoda is a class of Mollusca, comprising 832 known species (MolluscaBase, 2019). Although mollusks show sexual diversity (gonochorism, parthenogenesis, and hermaphroditism), it is well known that cephalopods generally only show gonochorism (Coe, 1944; Rocha et al., 2001). There are many morphological studies on cephalopods, focusing on the early gonadal development, sexual maturation process, and reproductive system structure (Arkhipkin, 1992; Avila-Poveda et al., 2009; Ikeda et al., 1991a, b; Laptikhovsky et al., 2003; Laptikhovsky and Arkhipkin, 2001; Melo and Sauer, 1999; Sauer and Lipiński, 1990). Additionally, the physiological mechanisms, including hormonal regulation, of sexual maturation in some species of cephalopods have been revealed using molecular biological methods (Cosmo and Cristo, 1998; Cosmo et al., 2001; Cristo et al., 2009; Kitano et al., 2017). On the other hand, morphological and physiological studies on gonadal sex differentiation in cephalopods are still limited to the common cuttlefish, *Sepia officinalis* (Montalenti and Vitagliano, 1946; Lemaire and Richard, 1970; Grasso and Di Grande, 1971; Lemaire, 1972), and the webfoot octopus, *Amphioctopus fangsiao* (Komorida and Yamamoto, 1993).

The kisslip cuttlefish, *Sepia lycidas*, is distributed throughout the eastern and southern China Sea, including in areas near southern Japan (Okutani, 2005; Wada et al., 2010). They have been shown to migrate to shallow coastal areas for mating and spawning during their breeding season (April to July in southern Japan; Natsukari and Tashiro, 1991). The cuttlefish is a suitable model species for morphological and physiological studies on the reproductive biology of cephalopods, because of the reported ease in breeding them in captivity (Domingues et al., 2006). *S. lycidas* is also well known as a marine resource, especially in Southeast Asia; thus, basic information on their reproductive biology is required for their conservation and sustainability. The aim of this study was to histologically clarify the gonadal sex differentiation process in *S. lycidas*.

2. Materials and methods

All experimental procedures involving animals were conducted in compliance with the Animal Care and Use Committee of the Institute for East China Sea Research, Nagasaki University, Japan (permit no. 15–06).

Wild parent *S. lycidas* were collected by fishing from the shallow coastal area of Nagasaki during their breeding season (April to July in southern Japan) in 2017 and 2018 (Wada et al., 2010). Individuals showed coupling behavior in a 500 L indoor circular polycarbonate resin tank; next, the mature females deposited their fertilized eggs on the rope placed in the tank as the spawning bed. The eggs were kept in actively

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aerated sea water in an indoor 30 L circular polycarbonate resin tank. Almost all individuals hatched at around 35 days after spawning (DAS). After hatching, juvenile cuttlefish were cultured with live mysid shrimp (*Neomysis intermedia*), then with *Palaemon pacificus*. As they grew, cuttlefish were fed twice daily on commercial frozen krill, then banded blue-sprat (*Spratelloides gracilis*), and Japanese horse-mackerel (*Trachurus japonicus*).

Before hatching, we dissected the eggs to obtain cuttlefish embryos. After hatching, prior to dissection, the juvenile cuttlefish were euthanized by decapitation, and the dorsal mantle length (DML) and body weight (BW) were measured. The gonads of 10–40 cuttlefish were collected at 14, 21, and 28 DAS, and 1, 10, 20, 30, 60, 90, and 150 days post hatching (DPH). The gonads were fixed in Bouin's solution, embedded in paraffin, cross-sectioned, and stained with a solution of Delafield's hematoxylin and 1% eosin, using standard methods for light microscopy. The developing stages of the oocytes and spermatocytes observed in this study were partially defined in accordance with Avila--Poveda et al. (2009) and Melo and Sauer (1999).

Sexual differences in the DML and BW of cuttlefish at the same growth stages during the experimental period were compared using the Mann–Whitney U test. Additionally, the sexual difference in the growth rate (GR) from 90 to 150 DPH was calculated using the mean BW (g day^{-1}), as described by Domingues et al. (2002).

3. Results

3.1. Sexually undifferentiated gonad

In one cuttlefish embryo at 14 DAS, an undifferentiated gonad, consisting of elliptical somatic cells and globular germ cells containing large nuclei, had already formed at a caudal site below the internal yolk sacks (Fig. 1a and b). The number of germ cells in the undifferentiated gonad gradually increased by mitotic proliferation from 14 to 21 DAS, and a blood vessel formed at 21 DAS (Fig. 1c). No sexual dimorphism in the gonadal morphology was observed at these embryonic stages.

3.2. Ovarian differentiation

In some cuttlefish embryos at 28 DAS and juvenile cuttlefish at 1 DPH, primary meiotic oocytes exhibited a large clear nucleus with many chromosomes distributed in the peripheral area of the gonad (Fig. 2a and b). Primary growth stage oocytes >20 μm in diameter, with densely stained cytoplasm, gradually appeared and developed in the ovary of juvenile cuttlefish from 10 to 30 DPH (Fig. 2c and d). The number of somatic cells and primary growth stage oocytes gradually increased in the central area of the immature ovary from 20 to 30 DPH (Fig. 2d). The large oocytes tended to be distributed in the outer periphery of the ovary, and follicle cell multiplication-stage oocytes, with many surrounding follicle cells, were observed; however, no oogonium was detected in the ovary at 60 and 90 DPH (Fig. 2e and f). Some of the somatic cells migrated from the interstitial tissues to the ovarian follicle area during this stage (Fig. 2g). At 150 DPH, early yolkless-stage oocytes with the follicular-fold complex, previously identified in the ovaries of cephalopods and thought to be a source of the yolk protein, were observed (Fig. 2h).

3.3. Testicular differentiation

In other individuals, although the gonad had gradually enlarged from the mitotic proliferation of germ cells, none of the meiotic germ cells were observed in the gonad from 28 DAS to 10 DPH (Fig. 3a and b). A seminiferous tubule consisting of a surrounding basement membrane and spermatogonia was first observed in the gonad at 20 DPH (Fig. 3c). Although spermatogonia in the seminiferous tubules gradually increased

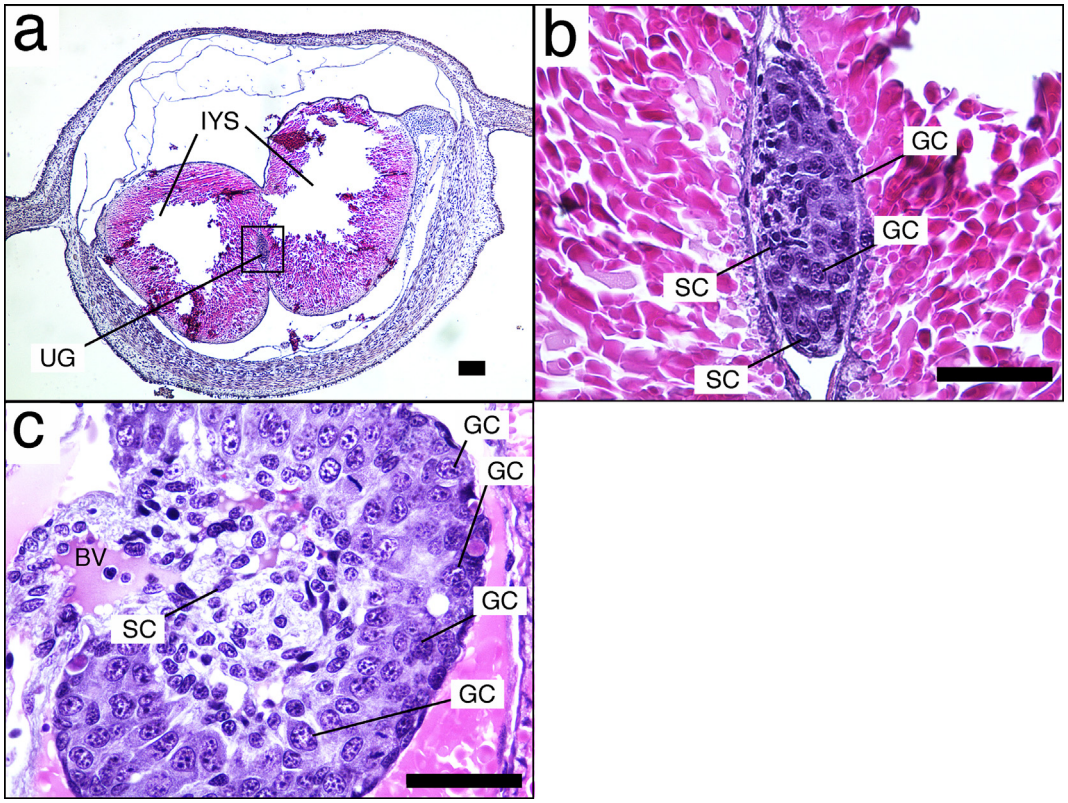

Fig. 1. Histological images of the undifferentiated gonad in *Sepia lycidas*. The undifferentiated gonad at 14 (a, low magnification; b, high magnification), and 21 days after spawning (DAS) (c). Inset square (a) indicates the area of the figure (b). UG, undifferentiated gonad; IYS, internal yolk sac; GC, germ cell; SC, somatic cell; BV, blood vessel. Scale bars = 100 μm.

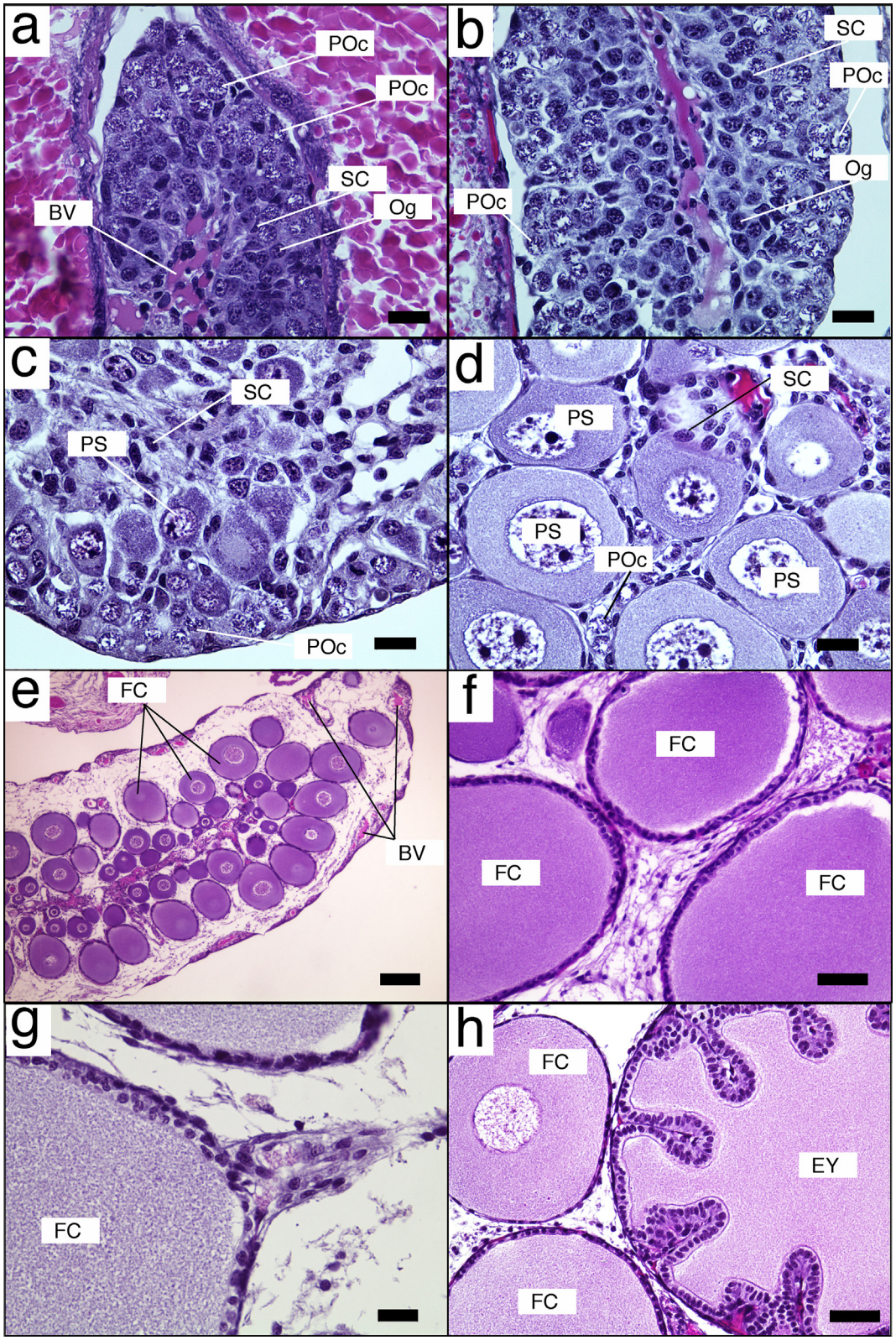

Fig. 2. Histological images of the ovary in *Sepia lycidas*. The immature ovary at 28 (a) days after spawning (DAS), 1 (b), 10 (c), and 30 (d) days post hatching (DPH), respectively. The developing ovary at 90 (e, low magnification; f, high magnification; g, showing the appearance of follicle cells), and 150 (h) DPH. POc, primary oocyte; Og, oogonium; PS, primary growth-stage oocyte; SC, somatic cell; FC, follicle cell multiplication-stage oocyte; EY, early yolkless-stage oocyte; BV, blood vessel. Scale bars = 20 μm (a, b, c, d, and g), 50 μm (f and h), and 200 μm (e).

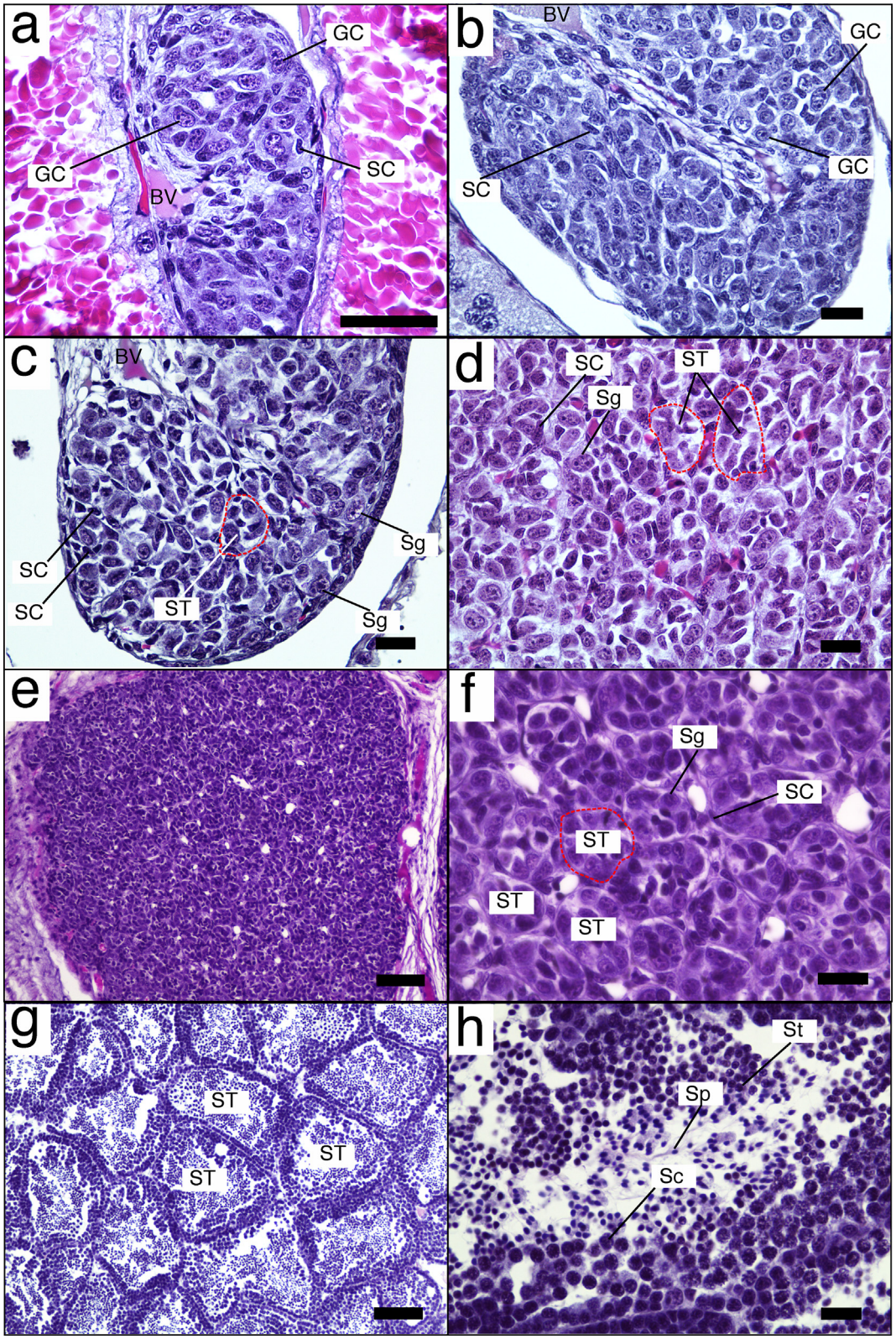

Fig. 3. Histological images of undifferentiated gonads and testes in *Sepia lycidas*. The undifferentiated gonad at 28 (a) days after spawning (DAS) and 10 (b) days post hatching (DPH). The immature testis at 20 (c), 60 (d), and 90 (e, low magnification; f, high magnification) DPH. The testis with active spermatogenesis at 150 (g, low magnification; h, high magnification) DPH. The red dotted lines in the figure (c, d, and f) indicates the seminiferous tubule. GC, germ cell; SC, somatic cell; ST, seminiferous tubule; Sg, spermatogonium; Sc, spermatocyte; St, spermatid, Sp, sperm; BV, blood vessel. Scale bars = 20 μm (b, c, d, f, and h), 50 μm (a), and 100 μm (e and g).

by mitotic division throughout the testes, spermatogenesis had not occurred even at 60 and 90 DPH (Fig. 3d, e, and f). Spermatogenesis, and a large amount of released sperm in the inner cavity of the seminiferous tubule, was first observed at 150 DPH (Fig. 3g and h). Neither the ovary nor the testis showed a duct-like structure during gonadal sex differentiation and development. A schematic diagram of the gonadal sex differentiation process in *S. lycidas* is shown in Fig. 4.

3.4. Growth and water temperature

The water temperature and cuttlefish size, age, sampling size, and gonadal status, are shown in Table 1 and Fig. 5. No significant difference ($P < 0.05$) was seen between the DML and BW, for male embryos, female embryos, or juvenile cuttlefish, at the same stages during the experimental period (Table 1). However, the GR of cuttlefish from 90 to 150 DPH was 1.04 g day^{-1} in females and 1.99 g day^{-1} in males.

4. Discussion

In this study, morphological gonadal sex differentiation during early ontogenesis in breeding *S. lycidas* was observed histologically. The results clearly showed that morphological ovarian differentiation is first characterized by the active meiotic proliferation of oocytes in the female gonads at around 28 DAS (DML = 6.90 ± 0.10 mm). No testicular morphological characteristics were observed in putative testes before 10 DPH. The seminiferous tubules previously reported as one of the morphological characteristics of the testes in the chokka squid, *Loligo reynaudii* (Sauer and Lipiński, 1990), first appeared in the testes at around 20 DPH (DML = 12.65 ± 0.37 mm). From these results, we conclude that in *S. Lycidas*, ovarian differentiation first arises at around 28 DAS (before hatching) with the appearance of meiotic oocytes, and testicular differentiation occurs by the formation of the seminiferous tubule at around 20 DPH. Our results are in accordance with reports that morphological ovarian differentiation arises faster than testicular differentiation by the appearance of oocytes in two other cephalopod species, *S. officinalis* and *A. fangsiao* (Lemaire and Richard, 1970; Grasso and Di Grande, 1971; Lemaire, 1972; Komorida and Yamamoto, 1993). There have been conflicting reports on the timing of gonadal sex differentiation in *S. officinalis*: it occurs either before (Grasso and Di Grande, 1971) or after hatching (Lemaire and Richard, 1970; Lemaire, 1972). In the present study, we clearly indicate that gonadal sex differentiation occurs before hatching in *S. lycidas*.

During the early gonadal development, we observed that the ovarian follicular cells formed the follicular-fold complex at around 150 DPH in *S. lycidas*. It was recently reported that this follicular-fold complex structure was the main site of vitellogenin, known as the main precursor of yolk protein synthesis in the swordtip squid, *Uroteuthis edulis* (Kitano et al., 2017). From these findings, we strongly suggest that oocyte maturation by the accumulation of vitellogenin begins at around 150 DPH in *S. lycidas*. Additionally, a large amount of released sperm was observed in the inner cavity of the seminiferous tubules in testes at 150 DPH. Thus, the testes had already reached sexual maturation at this age. However, female cuttlefish at the same age still had immature ovaries (at the beginning of vitellogenesis). These findings suggested that male *S. lycidas* reach sexual maturation faster than female *S. lycidas*, which is in accordance with reports on the Japanese common squid, *Todarodes pacificus* (Ikeda et al., 1991a and b).

Our results indicated that no significant differences were observed in the DML and BW between sexes at the same age during the early developmental period of *S. lycidas*. However, our results showed that female *S. lycidas* had a GR of around half that of males from 90 to 150 DPH. For the common cuttlefish, *S. officinalis*, it has been reported that females have a slower growth rate than males at the time of sexual maturation, as they invest more energy into reproduction (Domingues et al., 2002, 2006). From this, we hypothesized that for *S. lycidas*, the differentiated growth for each sex had possibly already begun at around

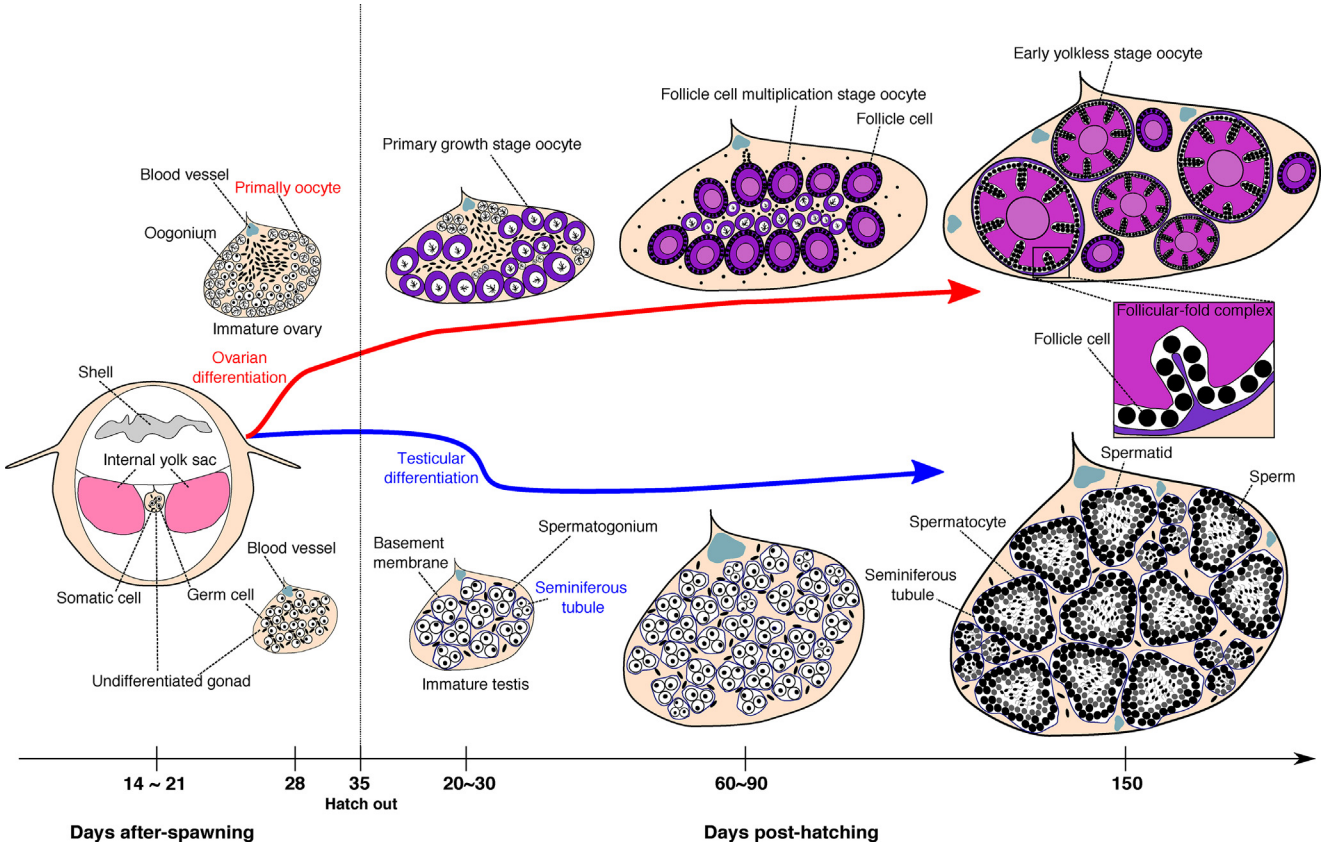

Fig. 4. Schematic diagram of the gonadal sex differentiation in *Sepia lycidas*.

Table 1
Age, sampling size, gonadal status, and size of *Sepia lycidas* from spawning to six months of age. DAS, days after spawning; DPH, days post hatching; *n*, number; DML, dorsal mantle length; BW, body weight.

Age	Sample size (n)	Gonadal status			DML (mm) mean ± SE			BW (g) mean ± SE		
DAS		Undifferentiated	Ovary	Testis	Undifferentiated	Female	Male	Undifferentiated	Female	Male
14	20	20	0	0	4.00 ± 0.00	-	-	0.03 ± 0.00	-	-
21	30	30	0	0	4.80 ± 0.13	-	-	0.05 ± 0.00	-	-
28	29	16	13	0	7.39 ± 0.16	6.90 ± 0.10	-	0.13 ± 0.00	0.12 ± 0.00	-
DPH										
1	42	22	20	0	7.92 ± 0.10	7.85 ± 0.16	-	0.14 ± 0.00	0.15 ± 0.00	-
10	44	26	18	0	11.13 ± 0.26	11.08 ± 0.21	-	0.37 ± 0.02	0.34 ± 0.02	-
20	35	4	15	16	12.70 ± 0.44	13.30 ± 0.30	12.65 ± 0.37	0.47 ± 0.04	0.58 ± 0.05	0.48 ± 0.04
30	39	0	18	21	-	18.75 ± 0.64	20.08 ± 0.64	-	1.24 ± 0.09	1.40 ± 0.09
60	20	0	11	9	-	36.00 ± 2.49	35.80 ± 2.44	-	5.84 ± 0.77	6.52 ± 0.94
90	10	0	6	4	-	81.83 ± 4.77	67.00 ± 3.32	-	51.94 ± 7.70	29.67 ± 3.67
150	10	0	7	3	-	108.29 ± 8.14	124.33 ± 8.09	-	114.57 ± 21.01	149.27 ± 23.14

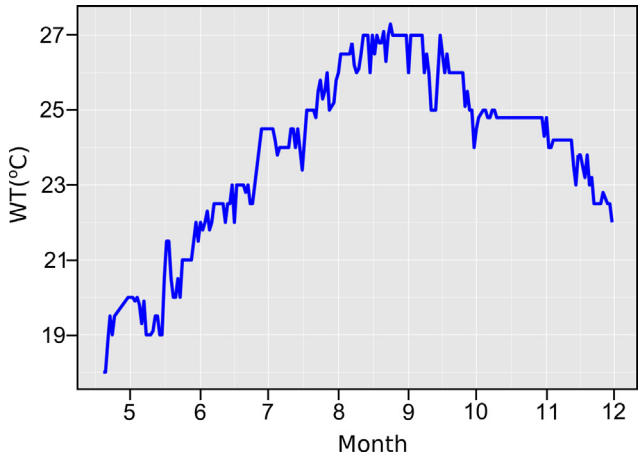

Fig. 5. Water temperature of the tank during the experimental period in 2018. WT, water temperature.

90–150 DPH, and the difference in sexual growth would be seen at the sexual maturation period, the same as for *S. officinalis* (Domingues et al., 2002, 2006). To confirm this prediction, future studies regarding the association between growth and sexual maturation in *S. lycidas* are needed. Additionally, it has also been reported that the length of *S. officinalis* embryonic development varies with water temperature (Domingues et al., 2006). Thus, when using the cuttlefish *S. lycidas* as a model species in future studies on reproductive physiology, the water temperature during their early development will have to be considered.

In conclusion, we clearly demonstrated that in cultured *S. lycidas*, ovarian differentiation occurs at around 28 DAS (before hatching) with the appearance of meiotic oocytes, whereas seminiferous tubules occur at around 20 DPH as the first characteristic of testicular differentiation. In future studies, we would like to investigate the effects of environmental (such as water temperature) and hormonal factors on gonadal sex differentiation and sexual maturation in *S. lycidas*.

Declarations

Author contribution statement

Ryosuke Murata: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Yuji Mushirobira, Kiyoshi Soyano: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Takeshi Fujita: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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