Physiological Studies on Gonadal Maturation of Fishes—I Sexual Difference in Composition of Plasma Protein of Ayu in Relation to Gonadal Maturation*

Katsumi AIDA**, Phan-Van-NGAN**, and Takashi HIBIYA**

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Differences by sex in the composition of plasma protein, its hormonal control and relationship between plasma protein and yolk protein of Ayu, *Plecoglossus altivelis*, were studied electrophoretically and immunologically. In immunoelectrophoresis, two new proteins (Fm-a and Fm-b) appeared in the female plasma with the commencement of vitellogenesis. Fm-a was composed of two fractions (fractions 9 and 11) in starch-gel electrophoresis, which increased in the maturing female plasma. In males, the appearance of new protein was not observed. Estradiol-17 β and estradiol-benzoate induced the dose-dependent synthesis of the new specific proteins in male fish, but testosterone, testosterone-propionate, hydrocortisone acetate and progesterone failed to produce the specific proteins. Ovarian extract caused three precipitin bands (Eg-a, Eg-b and Eg-c) in response to anti-maturing female Ayu plasma. Fm-a and Eg-a (a dominant fraction of ovarian extract) had the same antigenic component and binding substances such as calcium, lipid and polysaccharide. Eg-a was also composed of two fractions in starch-gel electrophoresis. In maturing females, plasma calcium increased simultaneously with the appearance of new specific protein and in estrogen-treated males (dose-dependent). These results suggest that in maturing female Ayu, new specific proteins, formed outside the ovary with the stimulation of estrogen, appear in the female plasma and then are taken up by the oocyte with the commencement of vitellogenesis.

It is well known that oocytes of teleosts accumulate a large amount of yolk substances which are necessary for development after fertilization. This yolk formation and accumulation of yolk is stimulated by gonadotropic hormone secreted from the pituitary gland¹⁻³). However, the systematic and detailed interpretation of the effects of gonadotropin on yolk formation and its accumulation has not yet been definitively resolved in teleosts.

Regarding the mechanism of yolk accumulation, the following three possibilities are conceivable as proposed by RAVEN⁴); (1) simple, elementary compounds are taken up and synthesized into yolk entirely by the oocyte itself; (2) substances semi-manufactured by the follicular cells are taken up and the final stages of synthesis are carried out by the egg cell itself; (3) substances pre-fabricated by some other cells of the body are taken up in finished form by the oocyte. Many histological and biochemical studies have been undertaken to clarify this mechanism,⁵⁻¹⁰) but there is still very little information on fishes.

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** Lab. of Fish Physiology, Fac. of Agr., The Univ. of Tokyo, Bunkyo-ku, Tokyo
(会田勝美・ファン・バン・ガン・日比谷 京: 東京大学農学部)

On the other hand, it is easily surmised that this enormous formation and accumulation of yolk substances in fish eggs probably induce some distinct metabolic alterations in the animal body. In fact, sexual difference in composition of plasma protein has been found in *Oncorhynchus kisutch*¹¹⁾, *O. nerka*¹²⁾, *Salmo salar*¹³⁾ and *S. gairdnerii* in¹⁴⁾ relation to gonadal maturation. However, despite the physiological interest and significance of this phenomenon, no further investigation has been carried out. The authors noticed that this difference was probably caused by a metabolic difference between the processes of vitellogenesis and of spermatogenesis and that this difference was a helpful clue in the clarification of the process of yolk accumulation. Therefore in the experiments presented in this paper, sexual difference in composition of plasma protein, its hormonal participation, and the relationship between plasma protein and yolk formation in Ayu, *Plecoglossus altivelis*, were studied as a first step towards elucidation of the mechanism of yolk formation in fishes.

Materials and Methods

Experiments Four experiments were carried out from 1968 to 1970 at the Freshwater Fisheries Research Laboratory, Hino, Tokyo (Experiments 1, 2 and 4), and the Fisheries Laboratory, the University of Tokyo, Maisaka, Shizuoka Prefecture (Experiment 3). The Ayu used in these experiments is well-known as an "annual fish", whose gonadal maturation is accelerated under a short photoperiod while suppressed under a long day-light condition.¹⁵⁾ Fish were brought from Lake Biwa and were fed with a commercial diet for Ayu throughout the experiments. However, Experiment 3 was carried out under conditions of fasting.

Experiment 1 was designed to examine the changes in composition of plasma protein following gonadal maturation under the natural photoperiod. Ten fish (5 of each sex, 9–55 g in body weight) were sampled from July to October on a weekly basis in 1968, and every 10 days in 1970.

Experiment 2 was undertaken to more precisely clarify the relationship between occurrence of sexual difference in composition of plasma protein and accumulation of yolk substances in ovarian eggs. One hundred immature fish (25–55 g in body weight) reared under a suppressive photoperiod for gonadal maturation (16 hours light—8 hours dark; 16L–8D), were brought to an accelerative condition (8L–16D) at the start of the experiment, whereupon 6–8 fish were sampled every other day until the 17th day.

In Experiment 3 and 4, the effect of several steroid hormones on the occurrence of sexual dimorphism in composition of plasma protein was studied. In Experiment 3, 110 immature males (20–40 g in body weight) reared under the suppressive condition (16L–8D) were divided into 11 groups at the start of the experiment, and kept in cages (50×50×60 cm) which were set in pounds (1×4 m), under the same photoperiod.

Experimental fish received intraperitoneal injections of steroid hormones dissolved or suspended in 0.2 ml of solvent (Table 3) on the first and fourth days, whereas controls received 0.2 ml of solvent without hormones, and were sampled on the seventh day. In Experiment 4, 40 immature fish were divided into 4 groups, each comprised of 10 fish (5 of each sex), which received intraperitoneal injections of 4 or 40 μ g estradiol-benzoate, and 20 or 200 μ g testosterone-propionate suspended in 0.2 ml of solvent (TEIKOKU ZOKI CO., LTD). Twenty mature fish were divided into 2 groups, each comprised of 10 fish (5 of each sex), which received intraperitoneal injections of 40 μ g of estradiol-benzoate and 200 μ g of testosterone-propionate. Injections and sampling were carried out under the same schedule as that of Experiment 3. Temperature of running water was 16–17°C in Experiments 1, 2 and 4, and 25–26°C in Experiment 3.

Analyses Blood was withdrawn and centrifuged at 3,000 r.p.m. for 15 minutes to separate plasma. Plasma protein was fractionated by starch-gel electrophoresis and agar-gel immunoelectrophoresis. Starch-gel (20.5 $\times$ 10 $\times$ 0.6 cm) was prepared in a plastic tray with 14.5% hydrolysed starch (JOKO SANGYO CO., LTD) in 0.045 M tris–0.001 M EDTA–0.025 M boric acid buffer (pH 8.5). For the bridge solution, 0.3 M boric acid–0.06 M NaOH was used. Electrophoresis was carried out horizontally at 4°C with a constant current of 2 mA/cm for 5 hours. Following electrophoresis, gels were horizontally sliced into 4 layers of 1, 2, 2 and 1 mm in thickness. The inner two slices of 2 mm thickness were stained with amidoblack 10 B or nigrosin and Sudan black B. Gels stained with amidoblack 10 B were analyzed on a densitometer at 620 m μ .

Immunoelectrophoresis was undertaken with a barbital buffer at pH 8.6 for 90 minutes. After electrophoresis, gels were made to react with rabbit anti-maturing female plasma which was produced by injecting a mixture of an equal volume of maturing female plasma and Freund Complete Ajuvant (IATRON LAB.) into the gluteal muscle of a male rabbit. OUCHTERLONY's double diffusion method was also employed.¹⁶⁾

Mature ovary or ovulated unfertilized eggs of Ayu were homogenized in cold 10% NaCl and centrifuged at 10,000 g for 30 minutes. The homogenate separated to three layers (lipid fraction, soluble fraction and residue). The soluble fraction was used as the crude egg extract in starch-gel or immunoelectrophoresis. The agar-gel plates reacted with antiserum were dried between filter paper forming agar-gel sheets and then stained with alizarin S for calcium,¹⁷⁾ PAS for polysaccharide and Sudan black B for lipid. Calcium in the plasma was determined by titration with EDTA.¹⁸⁾ Total amount of plasma protein was determined by the biuret reaction. For determination of developmental stages, gonads were fixed with ZENKER-formol and CARNOY's fixatives. Sections were cut to 4–5 μ , and stained with MAYER's acid hemalum-eosin, Azan and PAS.

Table 1. Total and fractional changes of plasma protein of Ayu reared under natural photoperiod in Experiment 1.

		1,2	3,4	5,6, (7)	Fractions (g/dl)			11	12, (13)	14	15	Total plasma protein(g/dl)*
Female	Jul. 22	0.04	1.27	1.22	0.78	0.48	0.11	0.69	0.27	0.04	4.90±0.16	
	Aug. 1	0.03	1.43	0.95	0.47	0.86	0.20	0.77	0.42	0.03	5.16±0.27	
	11	0.03	1.21	1.01	0.60	1.06	0.40	0.77	0.34	0.03	5.45±0.65	
	21	0.01	1.21	1.03	0.39	1.14	0.37	0.77	0.31	0.02	5.25±0.65	
	31	0.02	0.99	0.87	0.47	1.13	0.29	0.72	0.27	0.02	4.78±0.15	
	Sep. 10	0.02	1.19	1.41	0.50	1.73	0.50	0.93	0.31	0.03	6.62±0.85	
	20	0.02	1.03	1.25	0.61	1.37	0.41	0.75	0.38	0.03	5.85±0.66	
	30	0.02	0.86	1.16	0.43	1.24	0.34	0.58	0.25	0.02	4.90±0.32	
	Oct. 10	0.02	0.73	1.30	0.32	1.01	0.33	0.40	0.17	0.02	4.30±0.16	
	Jul. 22	0.03	1.26	1.04	0.76	0.60	0.16	0.84	0.23	0.03	4.95±0.32	
Male	Aug. 1	0.03	1.58	1.04	0.52	0.92	0.29	0.87	0.46	0.03	5.74±0.83	
	11	0.03	1.55	1.32	0.64	0.67	0.15	0.88	0.30	0.04	5.58±0.45	
	21	0.02	1.39	1.10	0.48	0.60	0.21	0.76	0.18	0.02	4.76±0.63	
	31	0.01	1.32	0.97	0.51	0.62	0.13	0.77	0.29	0.02	4.64±0.36	
	Sep. 10	0.02	1.64	1.31	0.57	0.74	0.22	0.85	0.60	0.03	5.98±0.47	
	20	0.03	1.71	1.58	0.58	0.58	0.22	0.73	0.48	0.04	5.95±0.62	
	30	0.02	1.18	1.17	0.56	0.47	0.13	0.54	0.40	0.03	4.50±0.48	
	Oct. 10	0.03	1.23	1.36	0.57	0.49	0.17	0.60	0.46	0.03	4.94±0.43	

* mean of five fish ± standard deviation.

Results

Fractional changes in relation to gonadal maturation In starch-gel electrophoresis, plasma protein of Ayu was composed of 12–15 fractions which were numbered as shown in Fig. 1. Fractions (F.) 6, 12 and 14 were lipoproteins. In some fish F. 7, 10 and 13 were recognized, but were not found in others. Therefore, these fractions were considered to be under genetic control. Total and fractional changes of plasma protein in Experiment 1 are summarized in Table 1 and gonadal development is presented in Fig. 2. A distinct increase of F. 9 and 11 occurred in the plasma of female Ayu sampled on August 10th and this continued until the time of death after spawning. On August 10th, the gonadosomatic index (GSI(%); wet weight of gonad/body weight, $\times 100$) was about 1% as shown in Fig. 2, and oocytes accumulated a small amount of yolk granules beneath the vitelline membrane. This sexual difference in electrophoretic pattern is illustrated

Fig. 1. Schema of electrophoretic pattern of Ayu plasma in starch-gel.

Fig. 2. Fluctuation of gonado-somatic index (wet weight of gonad/body weight, $\times 100$ (%)) of Ayu reared under natural photoperiod in Experiment 1. Empty circles, females; solid circles, males.

Fig. 3. Sexual difference in concentration of Fraction 9 and 11 in electrophoretic pattern of Ayu plasma. A, immature female; B, immature male; C, maturing female; D, maturing male.

in Fig. 3. Simultaneous with the increase, both F. 9 and 11 became stainable with Sudan black B (dye for lipid staining). Fraction 9 gradually decreased after having attained the highest value at the beginning of September. In females, F. 3 and 4, which were probably albumin, were present in a lesser amount than in males. A little later in September, the same decrease as found in F. 3+4 was recognized in F. 14, which was the slowest lipoprotein. Distinct changes in composition of male plasma were not found throughout gonadal maturation.

In immunoelectrophoresis, two new precipitin bands appeared in the female plasma sampled on August 10th and their presence continued until the time of death after spawning. The authors named these newly formed plasma proteins Fm-a and Fm-b as shown in Fig. 4. Their appearance was synchronized with the commencement of the increase of F. 9 and 11. Fm-a and Fm-b were considered to be the specific fractions in the plasma of maturing female Ayu. On the other hand, the appearance of newly formed proteins did not occur in male plasma.

The increase of F. 9 and 11, a lower level of F. 3+4 and 14, or an appearance of Fm-a and Fm-b in maturing female plasma are considered to be closely related with

Fig. 4. Sexual difference in immunoelectrophoretic pattern of Ayu plasma. A, immature female; B, immature male; C, maturing female; D, maturing male; E, maturing male; F, maturing female. 1. rabbit anti-maturing female plasma. 2. the same absorbed by maturing male plasma. Fm-a and Fm-b indicate newly formed proteins.

vitellogenesis, since the latter process in the ovary also started at the beginning of August in Ayu as shown in Fig. 2.

In Experiment 2, a slight increase in Fraction 9 was recognized in female plasma on the 11th day, and this tendency gradually became accelerated with time. A few oocytes at the primary yolk globule stage appeared for the first time on the 11th day, and rapidly

Table 2. Changes in hepato-somatic index, gonado-somatic index, Fraction 9 and development of eggs following change in photoperiod from a suppressive (16L-8D) to an accelerative maturation condition (8L-16D) in Experiment 2.

All values are shown as the mean of 3 or 4 fish.

Days	HSI (%)		GSI (%)		Fraction 9 (%)		Stages of Eggs (%)		
	female	male	female	male	female	male	Peri-nucleolus	Yolk vesicle	Primary yolk
0	1.66	1.54	0.30	0.04	6.6	6.1	95.6	4.4	0
1	1.67	1.51	0.32	0.05	6.8	6.4	93.8	6.2	0
3	1.64	1.65	0.32	0.07	5.9	5.8	84.5	15.5	0
5	1.57	1.43	0.46	0.06	7.1	6.3	72.0	28.0	0
7	1.42	1.19	0.40	0.08	5.5	6.1	74.9	25.1	0
9	1.45	1.55	0.47	0.17	6.6	6.3	69.2	30.8	0
11	1.59	1.15	0.82	0.22	9.1	5.5	60.5	38.8	0.7
13	1.61	1.62	1.05	0.42	17.8	7.2	44.8	38.5	16.7
15	1.51	1.28	1.31	0.81	20.2	7.3	47.5	28.5	24.0
17	1.47	1.17	1.91	1.20	18.7	6.7	40.6	22.4	37.0

increased as shown in Table 2. These results obtained in Experiment 2, also suggest a close relationship between the increase of F. 9 and the appearance of yolk granules in the oocytes. However, the increase of F. 11 had not occurred by the 17th day.

Effects of steroid hormones Among the four steroid hormones used in Experiment 3, only estradiol-17 β administration could induce an increase of F. 9 and 11 in immature male plasma. Two repetitive injections of 2 μ g estradiol-17 β at a two-day interval was the minimum dose for a recognizable increase. F. 9 and 11 increased with an increment in estrogen dosage (Fig. 5 and Table 3), and both fractions were stained with lipid staining. On the contrary, a decrease of F. 5+6, which was mainly due to a decrease of lipoprotein, was recognized. Total plasma protein also increased markedly, and this increase corresponded to the increment of F. 9 and 11. However, F. 3+4 and total plasma protein slightly increased with the treatment of hydrocortisone acetate.

In immunoelectrophoresis, it was also ascertained that Fm-a and Fm-b could be newly synthesized only with estrogen administration as shown in Fig. 6. Testosterone, hydrocortisone acetate and progesterone had no effect on the synthesis of new specific proteins. The precipitin bands became more distinct with the increase of dosage. This indicates that the synthesis of Fm-a and Fm-b is dose dependent. In Experiment 4, estradiol-benzoate stimulated the production of Fm-a and Fm-b in the plasma of not only immature males but also mature males and immature females. Testosterone-propionate had no effect on the appearance and disappearance of Fm-a and Fm-b.

Relationship between Fm-a and yolk protein. The crude ovary extract of mature female Ayu caused 3 precipitin bands (Eg-a, b and c) in response to anti-maturing female Ayu plasma as shown in Fig. 7. One of the precipitin bands, tentatively called Eg-a, was connected with that of Fm-a in natural maturing female (Fm-a-N) and Fm-a induced

by estrogen administration (Fm-a-E). This suggests that Eg-a contained the same antigenic component as that of Fm-a. The same reaction was recognized in ovulated unfertilized eggs. Moreover Eg-a and Fm-a were combined with calcium, polysaccharide and lipid. No other components of plasma and egg extract were combined with all these

Fig. 5. Effect of estradiol-17 β on starch-gel electrophoretic pattern of Ayu plasma. (A) control, (B) 0.2 μ g, (C) 2 μ g, (D) 20 μ g, (E) 200 μ g.

substances. After electrophoresis of estrogen-treated male plasma and egg extract in starch-gel, the sliced gel was cut into strips 5 mm in width and 6 cm in length, and then embedded in an agar-gel plate to react with antiserum. This double diffusion was carried out to determine which fractions in starch-gel electrophoresis were identical with Fm-a and Eg-a, respectively. This examination revealed that Fm-a was composed of F. 9

Table 3. Effects of steroid hormones on total plasma protein and its fraction in Experiment 3.

Treatment	1,2	3,4	5,6,(7)	8	9,(10)	11	12,(13)	14	15	Total plasma protein (g/dl)*
Control	0.02	1.22	1.02	0.57	0.45	0.23	0.69	0.23	0.02	4.45±0.11
Estradiol-17 β	0.01	1.26	1.04	0.64	0.56	0.28	0.66	0.28	0.01	4.74±0.42
Estradiol-17 β	0.01	1.47	0.97	0.70	0.95	0.50	0.72	0.37	0.01	5.70±0.52
Estradiol-17 β	0.02	1.59	1.04	0.55	1.56	0.70	0.79	0.33	0.02	6.60±0.58
Estradiol-17 β	0.01	1.42	0.65	0.47	3.12	2.04	0.77	0.61	0.01	9.10±1.23
Testosterone	0.02	1.09	1.02	0.47	0.49	0.23	0.71	0.27	0.02	4.32±0.60
Testosterone	0.01	1.37	1.04	0.44	0.51	0.19	0.58	0.21	0.02	4.37±0.46
Hydrocortisone acetate	0.02	1.21	1.22	0.58	0.41	0.31	0.66	0.15	0.02	4.58±0.52
Hydrocortisone acetate	0.01	1.71	1.06	0.84	0.64	0.33	0.76	0.26	0.02	5.63±0.44
Progesterone	0.01	1.07	0.99	0.54	0.38	0.23	0.60	0.23	0.01	4.06±0.40
Progesterone	0.02	1.24	1.13	0.61	0.48	0.37	0.61	0.20	0.02	4.68±0.40

Ethanol solutions of steroid hormones are dissolved or suspended in a solution of NaCl in such a manner that the final solvent concentration was 1.0% ethanol-0.8% NaCl.

* mean of five fish±standard deviation.

Fig. 6. Effects of steroid hormones on immunoelectrophoretic pattern of Ayu plasma in response to rabbit anti-maturing female Ayu plasma. (A) control; (B) estradiol-17 β , 0.2 μ g; (C) estradiol-17 β , 2 μ g (D) estradiol-17 β , 20 μ g; (E) estradiol-17 β , 200 μ g; (F) testosterone, 1 mg; (G) hydrocortisone acetate, 10 mg; (H) progesterone, 1 mg. Fm-a (or a) and Fm-b (b) indicate newly formed proteins by estrogen administration.

and 11, and Eg-a was also composed of two main components (lipoprotein) as shown in Fig. 8. However, these two lipoproteins in the egg extract showed less mobility than F. 9 and 11.

Fig. 7. Relationship between egg extract and Fm-a of maturing female and estrogen-treated male plasma. 1. rabbit anti-maturing female Ayu plasma. 2. the same absorbed by maturing male plasma.

Eg-a, b and c, precipitin bands of egg extract; Fm-a-N and b, precipitin bands of maturing female plasma; Fm-a-E, precipitin band of estrogen-treated male plasma.

Calcium in Plasma Calcium in female plasma increased in accordance with the development of ovary, and this exactly coincided with the increase of F. 9 and 11 and the appearance of Fm-a and Fm-b. The value of calcium in maturing females were 2-3 times higher than that of males (Table 4). In male plasma, the changes were not remarkable and displayed the same tendency as that of total plasma protein. Among the 4 steroid hormones used in Experiment 3, only estrogen could induce a marked increase of calcium in plasma. In the group treated with 200 μ g of estradiol-17 β , the mean value of 26.1 mEq/l attained was about 5 times higher than that of the control. Increase of

Fig. 8. (A) Identity of Fm-a in immunoelectrophoresis and Fractions 9 and 11 in starch-gel electrophoresis by the double diffusion method. (B) Identity of Eg-a in immunoelectrophoresis and fractions in starch-gel electrophoresis by the same method. a, lipid staining; b, protein staining; c, double diffusion in agar-gel. 1, rabbit anti-maturing female Ayu plasma. Arrows indicate Fm-a and Eg-a, respectively.

Table 4. Changes in calcium level of Ayu plasma following gonadal maturation and administration of steroid hormones.

All values are shown as the mean of five fish $\pm$ standard deviation.

		Calcium in plasma (mEq/l)		Treatment		Calcium in plasma (mEq/l)
		female	male			
Jul.	22	4.6 $\pm$ 0.35	4.6 $\pm$ 0.23	Control		5.4 $\pm$ 0.69
Aug.	1	6.5 $\pm$ 0.40	5.9 $\pm$ 0.50	Estradiol-17 β	0.2 μ g	6.2 $\pm$ 0.55
	11	9.8 $\pm$ 1.36	6.5 $\pm$ 0.88	Estradiol-17 β	2 μ g	7.7 $\pm$ 0.15
	21	10.7 $\pm$ 1.19	5.5 $\pm$ 0.44	Estradiol-17 β	20 μ g	14.2 $\pm$ 4.22
	31	11.9 $\pm$ 1.28	5.2 $\pm$ 0.66	Estradiol-17 β	200 μ g	26.1 $\pm$ 8.57
Sep.	10	14.8 $\pm$ 3.07	6.4 $\pm$ 0.49	Testosterone	10 μ g	5.5 $\pm$ 0.56
	20	11.5 $\pm$ 2.07	5.7 $\pm$ 0.65	Testosterone	1 mg	4.4 $\pm$ 1.25
	30	10.9 $\pm$ 1.53	5.1 $\pm$ 0.55	Hydrocortisone acetate	100 μ g	5.8 $\pm$ 0.94
Oct.	10	10.3 $\pm$ 1.08	5.2 $\pm$ 0.77	Hydrocortisone acetate	10 mg	6.8 $\pm$ 0.25
				Progesterone	10 μ g	6.8 $\pm$ 0.56
				Progesterone	1 mg	6.6 $\pm$ 0.58

calcium was dose-dependent and closely related with the increment of F. 9 and 11. Testosterone, hydrocortisone acetate and progesterone had no effect on the increase or decrease of calcium in plasma.

Discussion

Starch-gel electrophoresis has revealed that two specific plasma proteins (fast and slow) increased in the plasma of maturing female Ayu. This phenomenon found in the maturing female plasma, is generally similar to the results observed in salmonid fishes undergoing gonadal maturation.

In immunoelectrophoresis, it was ascertained that two precipitin bands (Fm-a and Fm-b) were specific for the plasma of maturing female Ayu, and that Fm-a was composed of two fractions (Fraction 9 and 11) in starch-gel electrophoresis. This indicates that F. 9 and 11 have the same antigenic component but electrophoretically different characteristics, and that the increase of F. 9 and 11 was caused by the addition of newly formed proteins to the pre-existing plasma proteins. Therefore, the authors named these newly formed proteins Female Specific Plasma Protein (FSPP).

Since FSPP was induced by estrogen administration in males and the amount was dependent on the dosage of estrogen, it is easily surmised that the synthesis is newly induced by estrogen in a certain organ possessing the potential to produce these proteins, and that the production is sensitive to the amount of estrogen. In other oviparous vertebrates, estrogen also induces the new synthesis of plasma proteins. URIST and SCHJEIDE¹⁹⁾ indicated that a new dense staining globulin was produced in response to estrogen in a teleost, amphibian and reptile, while two proteins were produced in a bird. FOLLET and REDSHAW²⁰⁾, however, found two newly formed proteins in the frog with acrylamide-gel electrophoresis. Since the increase of two specific proteins was recognized in the plasma of maturing female and estrogen-treated male Ayu, the authors concluded that at least two specific plasma proteins were newly synthesized with the commencement of vitellogenesis and the estrogen administration.

Fm-a and Eg-a had the same antigenic component and the same binding substances (calcium, polysaccharide and lipid) as illustrated in Fig. 7. Therefore, the physiological significance of these proteins seems to be considerably similar. In domestic fowl, phosvitin, lipovitellin and livetin are the main components of yolk proteins.²¹⁾ Phosvitin is a phosphoprotein (phosphorus=9.6%), and lipovitellin is a lipoprotein containing two components (α and β types; lipid=20%). On the other hand, livetin is the water-soluble protein of egg yolk, composed of many fractions which are ordinary plasma proteins such as albumin, low-molecular weight α^2 -glycoprotein, transferrin and γ -globulin.²²⁾ Furthermore, it has been made clear that the precursors of these yolk proteins exist in the plasma of laying hens and estrogen-treated pullets and cocks. In an

amphibian, yolk platelet is composed of two components, S and F.²³⁾ Component S is a phosphoprotein (phosphorus=8.4%, molecular weight 32,000) similar to phosvitin and Component F is a lipoprotein (lipid=17.5%, molecular weight 420,000) similar to lipovitellin in birds. It is also ascertained that the precursor (lipophosphoprotein) of yolk platelet proteins exists in the plasma of maturing female and estrogen-treated animals. Three main components seem to exist in teleostean eggs,²⁴⁻²⁸⁾ but the relationship between yolk components and the serum precursors have not been investigated in teleosts. Since Eg-a is a glycolipoprotein and consists of two components in starch-gel electrophoresis, this yolk component is probably of a lipovitellin type. Fm-a is considered to be the serum precursor of Eg-a, because it possessed the same antigenic property and binding substances as Eg-a. However, different mobilities of these proteins in starch-gel electrophoresis suggest that unknown changes occurred in the process of accumulation into the eggs. SCHJEIDE *et al.*²⁹⁾, indicated that yolk lipovitellin in birds exhibit a lower sedimentation constant and about half the molecular weight of their proposed serum precursor. However, the molecular weight of Fm-a seems to be smaller than that of Eg-a, since Fm-a was mobilized about two times faster than Eg-a in starch-gel electrophoresis. It is a interesting phenomenon that Fm-a and Eg-a are respectively composed of two fractions suggesting a relationship between monomer and dimer. A clarification of the biological and chemical significance of this phenomenon is one of the most important problems remaining for future investigation.

Since the appearance of yolk granules beneath the vitellin membrane closely coincided with the appearance of FSPP in female plasma and estrogen administration induced the production of FSPP in male plasma, it is suggested that FSPP produced outside the ovary is secreted into the blood and then transferred into the eggs. Estrogen probably controls this synthesis of FSPP. The same mechanism has been proven in birds and amphibians and the site of synthesis of new specific proteins is generally considered to be the liver.²⁹⁻³¹⁾ Therefore, the liver seems to be the most probable site of FSPP synthesis in teleosts. In fact, evidence which supports this hypothesis have been obtained in Ayu and will be reported in a subsequent paper.

It has been well known that the plasma calcium increases in mature female cod, puffer fish,³²⁾ killifish³³⁾ and goldfish,³⁴⁾ and estrogen-treated goldfish,^{34,35)} killifish³⁶⁾ and snake-head fish.³⁷⁾ The result on Ayu is similar to those of other fishes. Previous work has established that the plasma of laying birds contain a calcium-binding protein. The same protein is also produced in birds and amphibians by estrogen administration.^{38,39)} In teleosts, the appearance of calcium-binding FSPP was found in the plasma of maturing female and estrogen-treated male Ayu. Therefore, this appearance of calcium-binding protein seems to be a phenomenon common to oviparous vertebrates. Since the increase of calcium in Ayu plasma occurred simultaneously with the appearance of Fm-a, which

was combined with calcium, this elevated calcium level is probably due to the calcium bound with Fm-a. This is supported by the result of FOLLET and REDSHAW,²⁰⁾ who found that in the frog most of the increased calcium was of the protein-bound type. Calcium bound with new plasma protein, FSPP, may be accumulated in the ovarian eggs in the process of vitellogenesis.

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