Japanese Journal of Ichthyology
Vol. 34, No. 2 1987

魚類学雑誌
34巻2号 1987年

Induction of Female-Specific Serum Proteins in the Sand Lamprey, *Lampetra reissneri*, by Exogenous Estradiol-17β

Shoichi Fukayama, Akihiko Hara, Takahiro Matsubara
and Hiroya Takahashi

(Received December 2, 1986)

Abstract Responsiveness of the liver to estradiol-17β was examined immunologically by measuring the amount of female-specific serum proteins (FSSP) in the serum of the sand lamprey, *Lampetra reissneri*, injected with estrogen. FSSP synthesis was clearly induced in adult males and young adult lampreys of both sexes by two injections of estradiol-17β, at 200 μg/animal 3 days apart, while the same treatment was quite ineffective in ammocoetes larvae. Induction of FSSP synthesis was successful in some ammocoetes when estradiol-17β injections (200 μg/animal) were done once a week for three weeks. Discussions were made on the development of responsiveness of the liver to estrogen during ontogenesis of the sand lamprey.

In the sand lamprey, *Lampetra reissneri* (Dybowski), the female-specific serum proteins (FSSP), which may represent yolk precursor proteins (vitellogenin), are never detectable immunologically during the period of growth from the ammocoete stage to the inital stage of metamorphosis, first appear at the metamorphic stage 4, and reach the maximal level at the young adult stage (Fukayama et al., 1986). Ultrastructural changes of hepatocytes indicating an accelerated protein synthesis during and after metamorphosis in the lamprey are well correlated with the progression of vitellogenesis during that period (Fukayama, 1985).

It is well known that the synthesis of vitellogenin in the liver can be induced by estrogen treatment (Wallace and Selman, 1981; Wiegand, 1982). In the African clawed frog, *Xenopus laevis*, the induction of vitellogenin synthesis by estrogen is not possible in larvae before metamorphosis has not yet been commenced, and is first successful in those during metamorphosis (Follet et al., 1968; Skipper and Hamilton, 1979). May and Knowland (1981) have shown that the increased potential of vitellogenin production in the liver of the African clawed frog during metamorphosis has a relation to an increase in number of the nuclear receptors specific to estrogen, and that estrogen itself may act to increase the receptors. Few reports on these topics have hitherto been appeared in lampreys (Gorbamn, 1983) except the work of Turner et al. (1981). They showed that the number of estrogen receptors in the liver of female Pacific hagfish, *Eptatretus stouti*, was significantly abundant in vitellogenic females than in non-vitellogenic females.

In the present study, the induction of FSSP synthesis by the treatment with estradiol-17β was examined immunologically and ultrastructurally in ammocoetes, young adult specimens of both sexes and mature males of the sand lamprey. Induction of FSSP synthesis was successful even in some ammocoetes when estradiol injections were done repeatedly over a long period of days.

Material and methods

Ammocoetes, young adults of both sexes, and mature males of the sand lamprey, *Lampetra reissneri*, used in the present study were collected in the Ohno River, near Hakodate, Hokkaido, during the months from May to August in 1984 and 1985. They were transported to the laboratory immediately after capture and subsequently raised in plastic aquaria with aerated water under natural conditions of phtoperiod and temperature. Soft organic mud, about 5 cm deep, was sheeted on the bottom of the aquaria. The lampreys were acclimated to the laboratory conditions for at least a week before use in the following experiments.

Experiment 1. A total of 42 males with mature testes, 14.7±0.1 cm in mean total length, were used in this experiment. Twenty-one animals were

intraperitoneally injected each with 200 μg of estradiol-17β dissolved in olive oil, twice at an interval of three days. Blood samples were taken by amputating the tail of anesthetized animals on days 0, 2, 4, 6 and 8 setting the day of the second injection as day 0. The experiment was carried out at about 15°C under the natural photoperiod.

Experiments 2 and 3. A total of 18 young adult lampreys of both sexes and 17 ammocoetes measuring larger than 14 cm in total length were used in experiments 2 and 3, respectively. Several animals from each group (4 young adult females, 8 young adult males, 8 ammocoetes) were treated with estradiol by the same procedure as in experiment 1, and were sacrificed and bled six days after the second injection.

Experiment 4. A total of 20 ammocoetes larger than 14 cm in total length were used in this experiment. Ten ammocoetes were injected with 200 μg of estradiol per animal once a week for three weeks, and were sacrificed and bled six days after the third injection.

Control animals in each experiment were injected with the vehicle alone and subjected to blood samplings in a similar way to estradiol-treated animals. The last three experiments were performed under the natural photoperiod at water temperature from 18 to 25°C, using the animals of mean total lengths shown in Table 1.

Relative amounts of female-specific serum proteins (FSSP) of the sand lamprey were determined by the radial immunodiffusion technique of Mancini (Mancini et al. 1965), using an antiserum raised against the FSSP of the Japanese river lamprey, *Lampetra japonica*, as described in a previous paper (Fukayama et al., 1986). The amount measured was expressed as a percentage of the mean amount of FSSP determined in the young adult sand lampreys which served as controls in experiment 2 of the present study, except for experiment 1 in which the mean value measured on day 6 of estradiol treatment was taken as 100%.

The liver of the lampreys used in the present study was weighed for the determination of the hepatosomatic index (HSI; liver weight/body weight ×100), and was checked for ultrastructural changes of hepatocytes in the same manner as described earlier (Fukayama, 1985). The sex of the lampreys was determined by histological inspections of gonads preserved in Bouin's fluid.

Results

All of the male lampreys used in experiment 1 had testes filled with cysts containing mature spermatozoa. No trace of female-specific serum proteins (FSSP) was detected immunologically in their sera at the start of experiment. The injections of 200 μg of estradiol did not effect the appearance of FSSP in the serum of mature males

Table 1. Results of the treatment of young adult specimens of both sexes and ammocoetes of the sand lamprey, *Lampetra reissneri*, with estradiol-17β. Values represent the mean ±S.E.

Group	No. of animals	Mean total length (cm)	HSI*	No. of animals with FSSP	Relative serum level of FSSP
Experiment 2					
Estradiol-treated					
Female	4	15.5±0.4	0.96±0.11	4	279±23
Male	8	15.1±0.5	0.84±0.04	8	282±39
Control					
Female	3	15.3±0.7	1.01±0.04	3	100±5
Male	3	13.6±0.5	0.53±0.04	0	—
Experiment 3					
Estradiol-treated	8	15.1±0.4	0.59±0.03	0	—
Control	9	14.9±0.4	0.51±0.02	0	—
Experiment 4					
Estradiol-treated	10	16.2±0.4	0.64±0.04	4	61±15
Control	10	15.3±0.5	0.65±0.04	0	—

* HSI (hepatosomatic index, see the text).

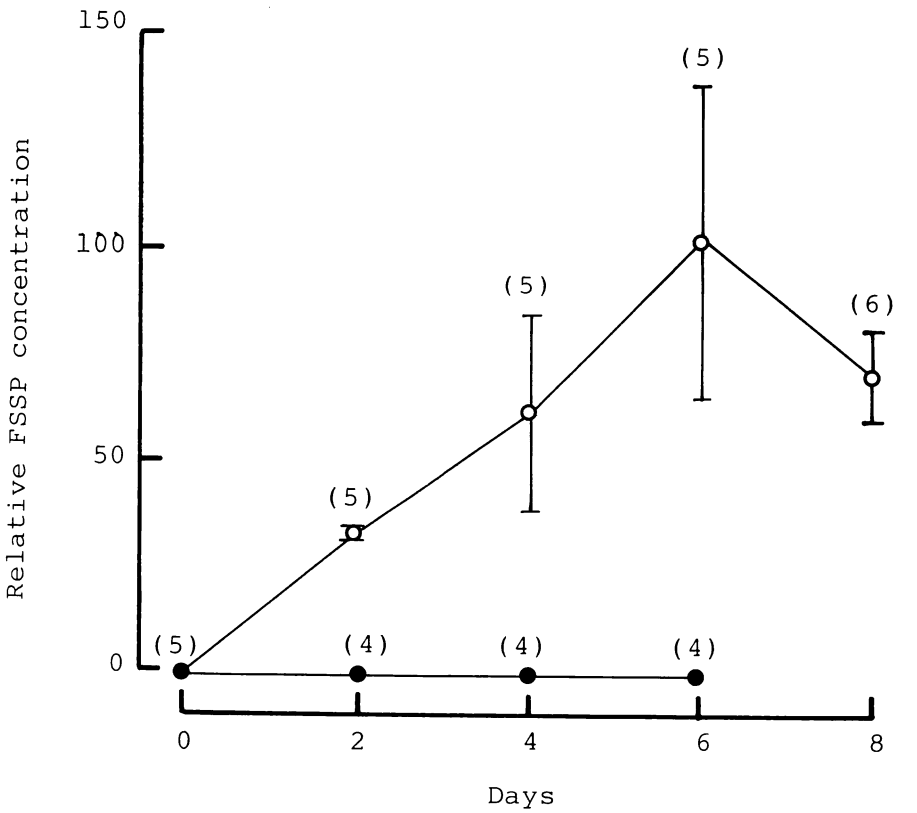
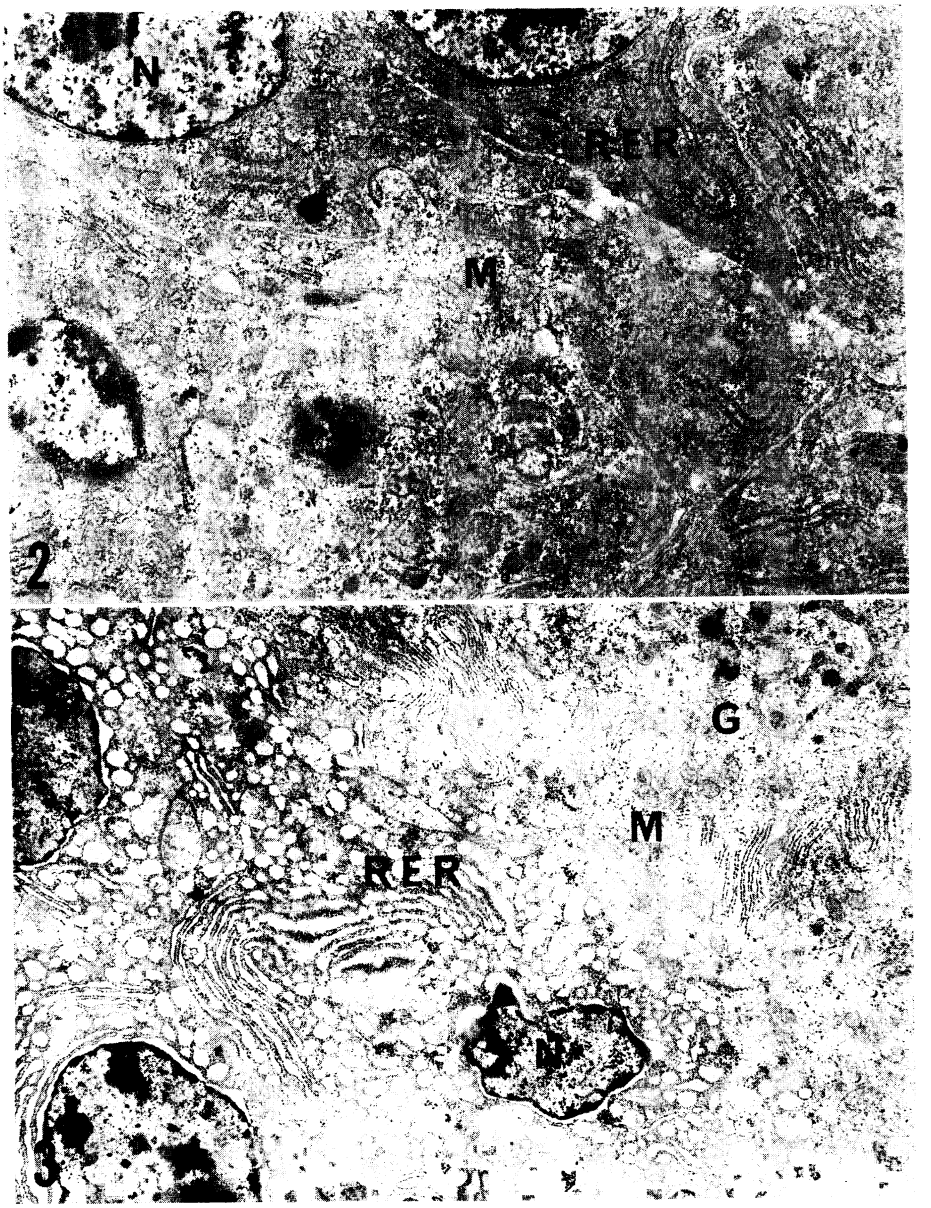


Fig. 1. Changes in serum FSSP concentration of estradiol-17β-treated (○) and control (●) males of the sand lamprey, *Lampetra reissneri*. Figures in parentheses indicate the number of specimens examined.

at least for four days postinjection. However, FSSP became detectable in all treated males as early as day 2 following the second injection of estradiol given on the fourth day after the first injection. Relative amounts of FSSP tended to increase to reach the maximum on day 6, though their change during the period from day 4 to day 8 was not significant statistically, due to wide ranges of individual variations of the amounts measured on these days (Fig. 1). FSSP was never detected in the sera of control males throughout the experiment.

The results of experiments 2–4 are presented in Table 1.

In young adult males of experiment 2, FSSP was present in a considerable amount in their sera six days after the second injections of estradiol, whereas it never occurred in those of the control group (Table 1). Females at the same stage of growth had already initiated the storage of yolk in ovarian oocytes, thus having a detectable amount of FSSP even in the control group at the start of experiment. Injections of estradiol into the female augmented FSSP level to be significantly higher than that measured in control females. No statistically significant difference was observed in the levels of FSSP between sexes of the treated animals. Hepatosomatic indices (HSI) were significantly smaller ($P<0.01$) in control males than in control females undergoing vitellogenesis, and apparently increased in males following estradiol treatment to reach the level found in vitellogenic females of the experiment (Table 1). An increase in HSI value in treated males accompanied an enlargement of hepatocytes, most of which were dark hepatocytes. Ultrastructurally, it was noticed that hepatocytes of treated males were characterized by having well-developed rough endoplasmic reticulum and Golgi apparatus (Fig. 3) compared with those of control males (Fig. 2).



Figs. 2 and 3. Electronmicrographs of hepatocytes of an estradiol-treated young adult male (Fig. 3) and of a control young adult male (Fig. 2). G, Golgi apparatus; M, mitochondrion; N, nucleus; RER, rough endoplasmic reticulum. Fig. 2. ×9650. Fig. 3. ×7500.

Ammocoetes used in experiments 3 and 4 had *either* ovaries with non-vitellogenic oocytes or testes with small spermatogonial cysts, except for one specimen of the estradiol-treated group of experiment 4 in which small gonial cysts predominated over non-vitellogenic oocytes in the gonad. None of them showed any sign of the commencement of metamorphosis throughout the experiments. In experiment 3, the treatment was carried out in the same manner as in experiment 2, and failed to induce the appearance of FSSP in any of the ammocoete treated ten days after the first injection. Ultrastructural aspects of hepatocytes of estradiol-treated ammocoetes were

hardly different from those of the cells of the control animals. In experiment 4, estradiol of the same dosage as in the other three experiments was given three times at prolonged intervals of a week. In this case FSSP was successfully detected, though smaller in relative amounts as compared with those in control young adults, in four out of nine ammocoetes with defined ovaries twenty days after the first injection (Table 1). No trace of FSSP was measured in six ammocoetes with ovaries in the control group. Significant differences in mean HSI values did not exist between ammocoetes with FSSP (0.66 ± 0.03) and those without FSSP (0.62 ± 0.06) in the estradiol-treated group. Ultrastructurally, however, there was a prominent development of the rough endoplasmic reticulum and Golgi apparatus in hepatocytes of the ammocoetes with FSSP as compared with that in the cells of the animals without FSSP, indicating stimulated protein synthesis of hepatocytes by the estradiol treatment.

Discussion

As reported previously (Fukayama et al., 1986), FSSP was detectable neither in the sera of larval specimens (ammocoetes) of the sand lamprey nor in those of the lampreys at the metamorphic stage 1. FSSP became detectable in the lampreys at the metamorphic stage 4 and reached its maximal level in those at the young adult stage just after metamorphosis. FSSP was never detected in the sera of males throughout their development from ammocoetes to mature adults.

It is well knwon in non-mammalian vertebrates that the synthesis of vitellogenin can be induced in the liver of immature females or males by treatment with estrogens (Wallace and Selman, 1981; Wiegand, 1982). Pickering (1976) has shown that, in the river lamprey, *Lampetra fluviatilis*, during spawning migration, implantation of estradiol-17β is effective in stimulating the production of yolk precursor proteins in both sexes. Similarly, in the Pacific hagfish, it has been reported that the synthesis of vitellogenin in the liver can be induced by the treatment with estradiol-17β (Yu et al., 1981). In the present experiment, FSSP was also clearly detected in all young adult specimens of both sexes six days after the second injection of estradiol-17β given at the fourth day at a dose of 200 μg per animal. Mean relative amount of FSSP was about three times as much as that of control females. Ultrastructural aspects of hepatocytes in young adult lampreys treated with estradiol were similar to those observed in the cells of young adult females at the time when the blood level of FSSP reached its peak (Fukayama, 1985).

FSSP could never be detected in ammocoetes injected with estradiol in the same manner as for the young adult lampreys, when examined ten days after the first injection. By contrast, induction of FSSP synthesis in ammocoetes was successful in four out of ten specimens receiving estradiol injections once a week for three weeks, when examined twenty days after the first injection, though mean relative amount of the induced FSSP in ammocoetes was significantly smaller than that measured in the estradiol-treated young adult lampreys. Furthermore, ultrastructural features indicating an activation of protein synthesis were seen in hepatocytes of ammocoetes with induced FSSP. These results suggest that the sensitivity to injected estradiol of the liver is very low in ammocoetes as compared with that in young adults. Sundararaj and Nath (1981) reported in the catfish, *Heteropneustes fossils*, that the intensity of vitellogenin synthesis in the liver was increased proportionally by repeated injections of estradiol-17β. They suggested that estradiol-17β not only induces the synthesis of vitellogenin but also primes the liver to respond to subsequent exposures to estradiol-17β. In addition, May and Knowland (1981) have shown, in the African clawed frog, that the sensitivity of the liver to estradiol is well correlated with the number of nuclear receptors in hepatocytes to estradiol-17β, and that estradiol-17β itself acts to increase the number. Turner et al. (1981) revealed that the number of estrogen binding hepatic nuclei of Pacific hagfish, was very high in adult vitellogenic females and sexually immature males. It is very likely that a similar explanation can be applicable to the results of the present study on the sand lamprey. In fact, blood levels of estradiol-17β were very low at ammocoete stage and gradually increased during metamorphosis (Fukayama, unpublished data). Thus, it is reasonable to assume that the low sensitivity of the ammocoetes liver to the treatment with estradiol-17β is due to low levels of endogenous estradiol-17β at the start of the experiment. This explanation seems

to be supported by the fact that the induction of FSSP synthesis became successful also in some ammocoetes when estradiol injections were done repeatedly over a longer period of days. On the other hand, it is likely that the liver of young adults of the sand lamprey have already acquired the ability to respond to estradiol-17β.

It is interesting to note that FSSP was undetected in six out of ten ammocoetes treated with estradiol. We reported the occurrence of a transitional gonad from ovary to testis during the larval stage in the sand lamprey (Fukayama and Takahashi, 1983). Thus, whether the difference in the responsiveness of the liver among ammocoetes has any relation to future sexes of the larvae is unknown at present.

Another important problem concerns whether the development of the sensitivity to estradiol of the liver may have a relation to metamorphosis that is to occur in the sand lamprey. To date, no report has dealt with the appearance of neotenic ammocoetes in any species of lampreys including the sand lamprey except for that of Zanandrea in *Lampetra zanandreai* (Zanandrea, 1956, 1957). It is well known that, in the Aftrican clawed frog, the induction of vitellogenin by estrogen treatment appears initially during metamorphosis (Follet et al., 1968; Skipper and Hamilton, 1979). Furthermore, Huber et al. (1979) have experimentally shown that, in the African clawed frog, the augmentation of the responsiveness to estrogen fails to appear when metamorphosis is suppressed, but readily appears after induction of metamorphosis by thyroxine. It has been demonstrated in some species of lampreys that blood levels of thyroid hormones change dramatically in the course of metamorphosis (Wright et al., 1978; Wright and Youson, 1978, 1980; Suzuki, 1982; Lintlop and Youson, 1983). This is true also for the present species of lampreys (Fukayama and Takahashi, 1983). Further investigations are needed to clarify the significance of thyroid hormones in the acquirement of responsiveness of the liver to estrogens to augment FSSP synthesis.

Literature cited

Follet, B. K., T. J. Nicholis and M. R. Redshaw. 1968. The vitellogenic response in the South African clawed toad (*Xenopus laevis* Daudin). J. Cell. Physiol., 72: 91–102.

Fukayama, S. 1985. Ultrastructural changes in the liver of the sand lamprey, *Lampetra reissneri* (Dybowski), during sexual maturation. Japan. J. Ichthyol., 32: 316–323.

Fukayama, S. and H. Takahashi. 1983. Sex differentiation and development of the gonad in the sand lamprey, *Lampetra reissneri*. Bull. Fac. Fish. Hokkaido Univ., 34: 279–290.

Fukayuama, S., H. Takahashi, T. Matsubara and A. Hara. 1986. Profiles of the female-specific serum protein in the Japanese river lamprey, *Lampetra japonica* (Martens), and the sand lamprey, *Lampetra reissneri* (Dybowski), in relation to sexual maturation. Comp. Biochem. Physiol., 84A: 45–48.

Gorbman, A. 1983. Reproduction in cyclostome fishes and its regulation. Pages 1–29 *in* W. S. Hoar, D. J. Randall and E. M. Donaldson, eds. Fish physiology. Vol. IX, Part A. Academic Press, New York.

Huber, S., G. U. Ryffel and R. Weber. 1979. Thyroid hormone induces competence for oestrogen-dependent vitellogenin synthesis in developing *Xenopus laevis* liver. Nature, 278: 65–67.

Lintlop, S. P. and J. H. Youson. 1983. Concentration of triiodothyronine in the sera of the sea lamprey, *Petromyzon marinus*, and the brook lamprey, *Lampetra lamottenii*, at various phases of the life cycle. Gen. Comp. Endocrinol., 49: 187–194.

Mancini, G., A. O. Carbonara and J. F. Hermans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry, 2: 235–254.

May, F. E. B. and J. Knowland. 1981. Oestrogen receptor levels and vitellogenin synthesis during development of *Xenopus laevis*. Nature, 292: 853–855.

Pickering, A. D. 1976. Effects of gonadectomy, oestradiol and testosterone on the migrating river lamprey, *Lampetra fluviatilis* L. Gen. Comp. Endocrinol., 28: 473–480.

Skipper, J. K. and T. H. Hamilton. 1979. *Xenopus* liver: Ontogeny of estrogen responsiveness. Science, 206: 693–695.

Sundararaj, B. I. and P. Nath. 1981. Steroid-induced synthesis of vitellogenin in the catfish, *Heteropneustes fossilis* (Bloch). Gen. Comp. Endocrinol., 43: 201–210.

Suzuki, S. 1982. Thyroid function in cyclostomes. Gunma Symp. Endocrinol., 19: 13–28.

Turner, R. T., W. W. Dickhoff and A. Gorbman. 1981. Estrogen binding to hepatic nuclei of Pacific hagfish, *Eptatretus stouti*. Gen. Comp. Endocrinol., 45: 26–29.

Wallace, R. A. and K. Selman. 1981. Cellular and dynamic aspects of oocyte growth in teleosts. Amer. Zool., 21: 325–343.

Wiegand, M. D. 1982. Vitellogenesis in fishes. Pages

136–146 *in* C. J. J. Richter and H. J. Th. Goos, eds. Reproductive physiology of fish. Pudoc, Wageningen.

Wright, G. M. and J. H. Youson. 1978. Serum thyroxine concentrations in larval and metamorphosing anadromous sea lamprey, *Petromyzon marinus* L. J. Exp. Zool., 202: 27–32.

Wright, G. M. and J. H. Youson. 1980. Variation in serum levels of thyroxine in anadromous larval lampreys, *Petromyzon marinus* L. Gen. Comp. Endocrinol., 41: 321–324.

Yu, J. Y.-L., W. W. Dickhoff, P. Swanson and A. Gorbman. 1981. Vitellogenesis and its hormonal regulation in the Pacific hagfish, *Eptatretus stouti* L. Gen. Comp. Endocrinol., 43: 492–502.

Zanandrea, G. 1956. Neotenia in *Lampetra zanandreai* (Vladykov) é l'endocrinologia sperimentale dei Ciclostomi. Boll. Zool., 22: 412–427.

Zanandrea, G. 1957. Neoteny in a lamprey. Nature, 179: 925–926.

(SF: Biological Evaluation Laboratory, Tokyo Research Institute, Kaken Pharmaceutical Co., Ltd., 2–28–8, Honkomagome, Bunkyo-ku, Tokyo 113, Japan; AH: National Research Institute of Aquaculture, Nansei, Mie 516–01, Japan; TM: Hokkaido Regional Fisheries Research Laboratory, Kushiro 085, Japan; HT: Department of Biology, Faculty of Fisheries, Hokkaido University, Minato-cho 3–1–1, Hakodate 041, Japan.)

エストラジオール処理によるスナヤツメの雌特異血清タンパクの誘導

深山昭一・原　彰彦・松原孝博・高橋裕哉

エストラジオール (E_2) で処理したスナヤツメ肝臓における雌特異血清タンパク (FSSP) の合成誘導を，免疫学的及び微細構造学的に調べた．実験に用いたスナヤツメは，アンモシーテス幼生，変態直後の若魚及び産卵期の雄成魚である．産卵期の雄成魚及び変態直後の若魚に，1尾あたり 200 μg 量の E_2 を，3日間隔で2回投与したところ，すべての処理個体の血中に FSSP が出現した．一方，アンモシーテス幼生に対する同様の処理は，FSSP 合成の誘導に対して全く無効であった．アンモシーテス幼生では，投与回数の増加，投与期間の延長により，一部の個体の血中に FSSP の出現を誘導し得た．粗面小胞体及びゴルジ体の発達を主徴とする肝細胞の微細構造上の変化は，FSSP が検出された個体においてのみ明瞭であった．以上の結果より，スナヤツメの肝臓の E_2 に対する FSSP 合成反応の能力は，成体と幼生とで異なることが明らかとなった．このようなスナヤツメ肝臓の FSSP 合成能にかかわる要因について考察し，さらにその合成能の発達をとくに変態現象と関連させて議論した．

(深山: 113 東京都文京区本駒込 2–28–8　科研製薬株式会社東京研究所生理活性研究室; 原: 516–01 三重県度会郡南勢町中津浜浦 422–1　養殖研究所; 松原: 085 釧路市桂恋 116　北海道区水産研究所; 高橋: 041 函館市港町 3–1–1　北海道大学水産学部淡水増殖学講座)