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POSSIBLE INVOLVEMENT OF SOMATOLACTIN IN THE REGULATION OF SEXUAL MATURATION AND SPAWNING OF MUGIL CEPHALUS

By

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ABSTRACT

*An antiserum to chum salmon somatolactin (SL) was used for immunocytochemical investigation of SL cell activity of **Mugil cephalus**, during gonadal cycle in both natural habitat and captivity. The SL-immunoreactive cells showed strong and specific immunoreactivity to anti-chum salmon somatolactin. The SL-immunoreactive cells showed an increase in the secretory and the synthetic activity during sexual maturation and spawning in natural habitat. SL cells were rather small and moderately immunoreactive in immature fish. They were enlarged and frequently more granulated during gonadal development. In addition, in late stages of maturation, comparatively larger and more degranulated cells were noted, also indicating an active release of SL granules. Prespawning females tended to have more enlarged SL cells with strong immunoreactivity than equivalent males. Degranulation, vacuolization and weak immunoreactivity of SL cells have occurred during spawning. The SL cells of **Mugil cephalus**, reared in captivity, appeared with high activity. This may be due to the low concentration of calcium in fresh water. The gradual stimulation of SL synthesis and release during sexual maturation and spawning of **M. cephalus** suggest that SL may be involved in the control of some steps of reproductive processes, such as steroidogenesis, calcium metabolism and energy mobilization.*

INTRODUCTION

Somatolactin (SL), a putative pituitary hormone, was isolated and characterized from pituitaries of a number of teleosts (Ono ***et al.***, 1990; Rand-Weaver ***et al.***, 1991a,b). Structurally this protein is related to growth hormone and prolactin (Ono ***et al.***, 1990; Takayama ***et al.***, 1991a,b). An antiserum raised against cod SL labeled the PAS-positive cells in the pars intermedia (PI) of several teleost species and homologous cells in the rainbow trout (Rand-Weaver ***et al.***, 1991b). Similar immunocytochemical localization of SL, using non-homologous antiserum, was observed in the PAS+ cells in the PI of ***O. niloticus*** (Mousa and Mousa, 1999a).

Although the biological function of SL is still largely unknown, recent studies have indicated that this pituitary hormone may participate in teleost reproduction, although SL has also been implicated in a number of other functions (for review, see Kaneko, 1996). Plasma SL levels increased during sexual maturation in coho salmon, ***Oncorhynchus kisutch***, reaching maximum levels at spawning (Rand-Weaver ***et al.***, 1992; Rand-Weaver and Swanson, 1993). Plasma SL levels were also found to be significantly higher in both mature male and female rainbow trout, ***Oncorhynchus mykiss***, than in immature fish (Rand-Weaver ***et al.***, 1995). In addition, gonadectomy of mature male Atlantic salmon, ***Salmo salar***, significantly reduced both plasma levels and the pituitary content of SL, suggesting that the mature testes have a stimulatory effects on the synthesis and release of SL (Mayer ***et al.***, 1998). This concurs with the finding that the SL cells in the pars intermedia are activated during the reproductive phase in ***Oncorhynchus*** (Olivereau and Rand-Weaver, 1994 a,b) and ***O. niloticus*** (Mousa and Mousa, 1999a). Further, SL exerts a weak steroidogenic activity on coho salmon gonadal tissue *in vitro* (Planas ***et al.***, 1992). In addition to being implicated in reproduction, SL levels have been shown to increase during the spawning migration of chum salmon, ***Oncorhynchus keta*** (Kakizawa ***et al.***, 1995a).

On the other hand, plasma SL levels in rainbow trout also increased in response to stress (Rand-Weaver ***et al.***, 1993). Activation of SL cells was observed in rainbow trout transferred from calcium-rich water to low-calcium water (Kakizawa ***et al.***, 1993). Also, the elevation of plasma SL, during acute stress and also during exhaustive exercise, in association with those of plasma cortisol, calcium, phosphate, and glucose levels, has suggested the involvement

of SL in calcium and phosphate metabolism, acid-base regulation, or energy mobilization in the stressed or exercised trout (Kakizawa ***et al.***, 1995b). Furthermore, involvement of SL in lipid metabolism has been suggested in cobalt variant of rainbow trout, which lacks SL-producing cells and accumulates much abdominal fat (Kaneko ***et al.***, 1993).

The present study has examined SL cell activity of ***Mugil cephalus*** at different stages of gonadal cycle in both natural habitat and captivity, to assess the possible actions of this hormone on sexual maturation and spawning of ***M. cephalus.***

MATERIAL AND METHODS

Fish Collection:

From El-Bardawill Lagoon (natural marine habitat; 10 mM Ca^{2+}) and El-Manzalah freshwater (0.8 mM Ca^{2+}) Fish Farm, mature females of ***M. cephalus*** (of standard length > 28 cm and body weight: 700-1500 g.) were collected alive at intervals of about one month throughout the year (1998). However, during the prespawning and spawning season (September and October), fish were collected at intervals of about 15 days to ensure that all stages of gonad maturation were included. Gonadosomatic index (GSI= (Gonad weight/Gutted fish weight) H 100), oocyte diameter, and histological appearance were used as indices of different maturity stages. Five stages of testicular activity and seven stages of ovarian maturation were recognized (Mousa, 1994; Mousa and Mousa, 1997; 1999b). The ripening stage (VI) of females was noticed to occur immediately before spawning. No ovaries could be found in the fish in this stage whether those collected from natural habitat or captivity. However, this stage was induced experimentally by injection of hormones (7 pituitary glands of ***M. cephalus*** + 4500 I.U. of human chorionic gonadotropin) into prespawning females (Mousa, 1994).

Histological and histochemical methods:

Prior to dissection, the fishes were anesthetized in a solution (100 mg/l.) of tricaine methanosulfonate (MS222, Sandoz) and then

decapitated. The pituitary gland, attached to the brain, was fixed in Bouin's fluid for 24 hr. The fixed brain and pituitaries were thereafter dehydrated through graded ethanol solution, cleared and embedded in paraplast (M.P. 56-58 °C). Consecutive median sagittal sections of the brain and pituitary gland were made at 5 µm thickness. For each specimen, selected sections were stained with Periodic Acid Schiff-Lead hematoxylin-Orange G (PAS-PbH-OG): lead hematoxylin "PbH" (McConail. 1947), combined with PAS-OG (Pearse, 1949).

Immunocytochemical Procedures:

Antisera:

Antisera to chum salmon somatolactin (SL. Lot No. 8906) chum salmon somatotropin (GH. Lot No. 8208) and chum salmon prolactin (PRL Lot No. 8502) were obtained from Dr. H. Kawauchi (School of Fisheries, Japan).

Immunocytochemical reactions:

Immunocytochemical staining for the sections of the pituitary gland was generally performed with a vectastain ABC (Avidin-biotin peroxidase complex) Kit (Vector Laboratories) as described previously (Mousa and Mousa, 1999). In brief, sections were deparaffinized in xylene, rehydrated through graded ethanol, washed in phosphate-buffered saline (PBS; pH 7.4) for two times 10 min each. All incubations were done at room temperature and PBS was used for washing after each step. Sections were incubated with the antiserum to chum salmon prolactin, chum salmon somatotropin or chum salmon somatolactin for 12 hr. Thereafter, the sections were incubated with the biotinylated secondary antibody (Vector Laboratories) for 1h. and with avidin- biotin-conjugated peroxidase for 45 min. Finally, the sections were washed and stained with 3`, 3`- diaminobenzidine tetrahydrochloride (DAB) (Sigma) including 0.01 % H_2O_2 in 0.05 M Tris-buffered saline (pH 7.6) for 5 min. After the enzyme reaction, the sections were washed in tap water, dehydrated in alcohol, cleared in xylene and mounted in DPX.

Since a heterologous SL antiserum was used in this study, it was essential to gain a perspective on the specificity of this antiserum. To prove the specificity of staining, various controls were used. In some, primary antiserum preabsorbed with an excess of antigen. Also primary or secondary antibody or avidin-biotin- peroxidase complex was omitted. In addition, to verify that the SL antiserum was not reacting with the presumptive PRL and GH cells in the pars distalis, it was important to demonstrate that both PRL and GH antisera did not recognize SL-related immunoreactivity in the pars intermedia.

RESULTS

Using specific antibody for the chum salmon somatolactin, immunocytochemistry was employed to investigate the cellular activity of this hormone in the pituitary gland of ***M. cephalus*** during sexual maturation and spawning.

Immunostaining of somatolactin (SL) cells in the pituitary gland:

The PAS+ cells of the pars intermedia (PI) (Fig. 1a) showed strong and specific immunoreactivity to anti-chum salmon somatolactin (SL) (Fig. 1b), which gave no cross reaction with any of the other cell types (Fig. 1b and 1c). Preabsorption of SL antiserum with purified antigen eliminated all staining reaction, confirming the specificity of the antiserum (Fig. 1d). In addition, the SL-immunoreactive cells reacted negatively with both antiserum of PRL and antiserum of GH (Figs. 1e and 1f).

Cyclic changes in the somatolactin (SL) cells:

The number, size and intensity of the immunoreaction of the SL cells represent seasonal variations, being concomitant with the development of the gonads and spawning as illustrated in figures (2), (3) and (4).

The cross-sectional area of the SL cells varied according to the section level so that an accurate evaluation of their volume was unrealistic. Similarly, their distribution among cell cords of the PI was often heterogeneous and a quantification of their relative number would be meaningless. However, the synthetic activity of SL cells was reflected by the intensity of the

and with variable immunoreactivity (Fig. 3b and 3c). Most of the SL cells exhibited accumulation of immunoreactive granules and some of them appeared to empty their secretory contents as indicated by their degranulated appearance. During the late-vitellogenic stage, the SL cells showed faint or moderately immunoreactivity, and most of them became degranulated on account of the discharge of their secretory contents (Fig. 4a). Also, some of SL cells had a revival of immunoreactive granules. Thereafter, in the prespawning fish, the SL cells hypertrophied, showed strong immunoreactivity and appeared to be highly granulated (Fig. 4b). However, in experimentally induced mature females with ripe ovaries, which acclimatized to saline water, most of the SL cells appeared to be degranulated with faint or moderately immunoreaction on account of the heavy loss of secretory granules (Fig. 4c) and some of SL cells had granulated appearance. Later on, by the approach of postspawning phase of the ovary, the immunostaining intensities and size of SL cells appeared greatly reduced compared to that of prespawning female (Fig. 4f). In this stage the SL cells became degranulated.

B) In captivity (fresh water):

In the previtellogenic and vitellogenic females, the number of SL cells appeared greatly increased compared to that observed in natural habitat (Figs. 3d, 3e and 3f). The SL cells exhibited high synthetic activity as reflected by the strong immunoreactivity (Figs. 3d, 3e, 3f and 4d), but the secretory activity was similar to that of natural habitat. However, during the resorption of the ovary (atresia), the immunoreactivity of the SL cells appeared greatly reduced compared to that of the prespawning stage in natural habitat (Fig. 4e). Most of SL cells of fish with degenerating ovaries became degranulated with rarefied immunoreactive granules.

DISCUSSION

The present study investigated SL cell activity in the pituitary gland of ***M. cephalus*** at different stages of gonadal cycle in both natural habitat and captivity. This study was carried out by using an antiserum produced against chum salmon somatolactin (SL) and showed that SL is synthesized in the PAS+ cells in the pars intermedia (PI) which is characteristically located along the neurohypophyseal/PI border. Amino acid sequence alignment revealed that teleost SLs exhibit a high percentage of identity (Takayama ***et al.***, 1991b; Rand-weaver and Swanson, 1993; Johnson ***et al.***, 1997; Yang ***et al.***, 1997). The

gene structure of chum salmon (***Oncorhynchus keta***) SL has been described (Takayama ***et al.***, 1991a) and its similarity to cod SL (Rand - Weaver ***et al.***, 1991a) is approximately 82 % and the identities of their amino acid sequences were between 73 and 81 % (Takayama ***et al.***, 1991b). This highly conserved nature of teleost SLs, may explain why SL-containing cells are localized with non-homologous antiserum. Although somatolactin (SL) has been shown to be related to GH and PRL (Ono ***et al.***, 1990; Rand-Weaver ***et al.***, 1991b), the regional localization of SL in the pituitary of ***M. cephalus*** is clearly different from these other two hormones which are produced by the pituitary pars distalis. In addition, the present study indicated that anti-chum SL gave no cross reaction with both GH and PRL secreting cells and the SL-immunoreactive cells reacted negatively with both antiserum of GH and antiserum of PRL. Similar immunocytochemical localization of SL, using non-homologous antiserum, was observed in the PAS + cells in the PI of several teleosts including ***Pleuronectes flesus, Poecilia latipinna, Heteropneustes fossilis, Fundulus heteroclitus and Anguilla anguilla*** (Rand-Weaver ***et al.***, 1991a), ***Oreochromis mossambicus*** (Dores ***et al.***, 1996), ***Sparus aurata*** (Villaplana ***et al.***, 1997) and ***O. niloticus*** (Mousa and Mousa, 1999a), as well as the chromophobic cells present in the pars intermedia of ***Oncorhynchus mykiss, O. nerka*** and ***O.keta*** (Kaneko ***et al.***, 1993; Olivereau and Rand-Weaver, 1994a).

In the present study, the synthetic and secretory activity of the SL-immunoreactive cells in the PI of ***M. cephalus*** showed an increase during sexual maturation and spawning. Olivereau and Rand-Weaver (1994a, b) obtained a similar immunocytochemical observations in ***Oncorhynchus nerka*** and ***O. keta*** and Mousa and Mousa (1999a) in ***O. niloticus***. They observed an increase in number, size and activity of SL cells during sexual maturation of these teleost species.

In both males and females, the increase of SL cell activity is correlated to the initial stage of sexual development according to the gonadal weight or volume increase. In general, the synthetic activity of SL cells of ***M. cephalus*** reared in captivity was rather high. This may be due to the dark background (turbidity), increase of stress or the low concentration of calcium in fresh water. Similar findings suggest that SL cells are activated by low calcium concentration in ambient water in rainbow trout, possibly suggesting a hypercalcemic action of SL (Kakizawa ***et al***, 1993). No differences are apparent between male and female pituitaries of immature or early maturing mullet, in

both natural habitat and captivity, but in late stages of maturation, the difference in SL cell activity was more abundant. As spermatogenesis progressed (rapid spermatogenesis), the SL cells become enlarged and granule synthesis is evident, since the strong immunoreaction of them was observed. The occurrence of degranulated and small size SL cells in ripe males suggests that release may be stimulated as soon as spawning starts. The high level of testosterone, recorded by Mousa (1994) and Zaki ***et al***. (1995), during ripening stage of ***M. cephalus*** males, had presumably caused a stimulatory effect on the release of SL. This concurs with the finding that, in ***Salmo salar***, the mature testes have a stimulatory effect on the synthesis and release of SL (Mayer ***et al***., 1998). The release seems to be further increased in both freshwater ripe males, acclimatized to saline water, and postspawning (spent) fish. In these fishes, the SL cells appeared greatly reduced in number, degranulated and with weak immunoreaction. Similar immunocytochemical observations were obtained in ***O. niloticus*** (Mousa and Mousa, 1999a).

During the late-vitellogenic stage, the secretory activity of the SL cells increased as indicated by their degranulated appearance. The gradual increase of SL release (secretory activity) during vitellogenesis is highly correlated with estradiol levels observed by Mousa (1994) and Zaki ***et al***. (1995). In addition, the present immunocytochemical results are in accordance with biochemical studies, in ***Oncorhynchus kisutch*** which showed that SL stimulates gonadal steroidogenesis *in vitro* (Planas ***et al***., 1992). Also, Rand-Weaver ***et al***. (1992, 1995) found that in ***O. kisutch*** during the period of gonadal growth, plasma SL levels increased and were highly correlated to estradiol levels in females and 11-ketotestosterone levels in males. In addition to being implicated in gonadal steroidogenesis, SL may be has a hypercalcemic action during gonadal maturation. In female teleosts, sexual maturation is driven by a gonadotropin-induced increase in plasma estradiol-17β (E_2) levels (Nagahama, 1987). E_2 stimulates the liver to produce vitellogenin (VTG) which is a yolk protein precursor. The VTG is released to the circulation and as VTG binds calcium, total plasma calcium levels increase. VTG is subsequently sequestered by the oocytes, processed and stored as nutrition for the embryo. During this period of VTG production the calcium demand increases, which can be met by mobilization of calcium from internal and/or external sources. The present observed gradual increase of SL release during vitellogenesis is highly correlated with the calcium demand, as the calcium accumulation in the female gonad increases during this period. A hypercalcemic action of SL has been

indicated in previous studies: SL cells are activated by low environmental Ca^{2+} levels and plasma SL levels are elevated in association with the increase in plasma Ca^{2+} levels in stressed and exercised fish (Kakizawa ***et al.***, 1993, 1995a, 1996). Although the activation of SL cells during ovarian maturation development suggests a hypercalcemic action of SL, it is likely that other hormones, such as prolactin, stanniocalcin and calcitonin, are involved in calcium homeostasis in teleosts (Hirano, 1989; Copp and Kline, 1989; Wendelaar Bonga and Pang, 1991). It is clearly necessary to examine the role of SL in calcium homeostasis in the context of other calcemic hormones.

In prespawning females, SL cells were enlarged and frequently more granulated, suggesting high synthetic activity of SL. Later on, by the approach of postspawning phase of the ovary, the SL cells were degranulated, indicating an active release of SL granules during spawning migration. This may indicate the involvement of SL not only in gonadal deveopment but also in energy mobilization related to reproduction, since the biological events, during spawning migration, concerned with reproduction require a great deal of energy. Activated SL cells identified immunocytochemically, were seen also in spawning ***Oncorhynchus nerka***, ***O. keta*** and ***O. tshawytscha*** (Olivereau and Rand-Weaver, 1994a,b). In addition, the present immunocytochemical results received a good support from biochemical studies, which showed that SL levels have been shown to increase during the spawning migration of chum salmon ***Oncorhynchus keta*** (Kakizawa ***et al.***, 1995b).

The gradual stimulation of SL synthesis and release during sexual maturation and spawning of ***M. cephalus*** suggests that SL may be involved in the control of some biological events concerned with reproduction, such as steroidogenesis, calcium metabolism and energy mobilization. The possible cause-result relationship between SL and each of these biological events should be examined independently in attempts to clarify the definitive function(s) of SL.

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EXPLANATION OF FIGURES

Fig (1): Sagittal sections of the pituitary gland of ***M. cephalus.***

a- Stained with PAS-PbH. Note the cell types in the PI: the PAS-positive cells (arrows) and PbH-positive cells (arrowheads). X1000.

b- and c) Immunostained with anti-chum salmon SL. Note the strong and specific immunoreactivity of the SL cells (b, X1000) which distributed throughout the PI (c, X50).

c- Preabsorping of antibody with purified chum salmon SL eliminated staining in SL- immunoreactive cells (X50).

d- Immunostained with anti-chum salmon prolactin antiserum. PRL positive cells are restricted in the rostral pars distalis (X50).

e- Immunostained with anti-chum salmon growth hormone (anti-chum GH) antiserum. The GH-positive cells are distributed throughout the proximal pars distalis (X50).

Fig (2): Sagittal sections of the pituitary gland of ***M. cephalus*** immunostained with anti-chum salmon SL antiserum (X1000). .

a- Immature male obtained from natural habitat (saline water). Note the SL cells having small size and small amount of cytoplasm and exhibiting strong immunoreaction.

b- Male obtained from natural habitat, during the period of stimulating spermatogenesis. The SL cells are large in size and having granulated (arrows) degranulated (arrowheads) and vacuolated (v) appearance.

c- Male obtained during the period of rapid spermatogenesis from natural habitat. Note the hypertrophied SL cells with strong immunoreaction.

d- Immature male obtained from captivity (fresh water). The SL cells are many in number and exhibiting strong immunoreactivity.

e- Male obtained from captivity at stimulating spermatogenic stage. Most of the SL cells are granulated (arrows) and the other are degranulated (arrowheads).

f- Male obtained from captivity at rapid spermatogenic stage. Note the strong immunoreactivity of the granulated SL cells (arrows). Also, some of degranulated SL cells (arrowheads) are present.

g- Ripe male obtained from natural habitat. Note most of the SL cells are degranulated (arrow heads) with scarce immunoreactive granules and some of them are granulated (arrows).

h- Ripe male obtained from captivity and acclimatized to saline water. The SL cells are decreased in number and degranulated. Note the rarefied of immunoreactive granules.

i- Spent male obtained from natural habitat. The SL cells are small in size and number, and degranulated. Note some of the SL cells with moderately immunoreaction.

Fig (3): Sagittal sections of the pituitary gland of ***M. cephalus*** immunostained with anti-chum salmon SL antiserum (X1000).

a- Immature female (previtellogenic) obtained from natural habitat (saline water). Note the gradual accumulation of the immunoreactive granules in the SL cells.

b- Female with early vitellogenic ovary obtained from natural habitat. The SL cells exhibited strong immunoreaction.

c- Female with mid-vitellogenic ovary obtained from natural habitat. The SL cells are stained intensely as those of the early vitellogenic stage. Note the degranulated appearance of some SL cells (arrowheads).

d- Previtellogenic female obtained from captivity (fresh water). The SL cells are increased in number. Note accumulation of immunoreactive granules in most of the SL cells (arrows) and some cells are degranulated (arrowheads).

e- Female with early vitellogenic ovary obtained from captivity. The size of the SL cells and the accumulation of the immunoreactive granules increased than that of previtellogenic stage.

f- Female with mid-vitellogenic ovary obtained from captivity. Note the SL cells are exhibited variable immunoreactivity as that of natural habitat.

Fig (4): Sagittal sections of the pituitary gland of ***M. cephalus*** immunostained with anti-chum salmon SL antiserum (X1000).

a- Female with late-vitellogenic ovary obtained from natural habitat. The SL cells are decreased in number. Note most of the SL cells are degranulated with scarce immunoreactive granules and some of them are granulated (arrows).

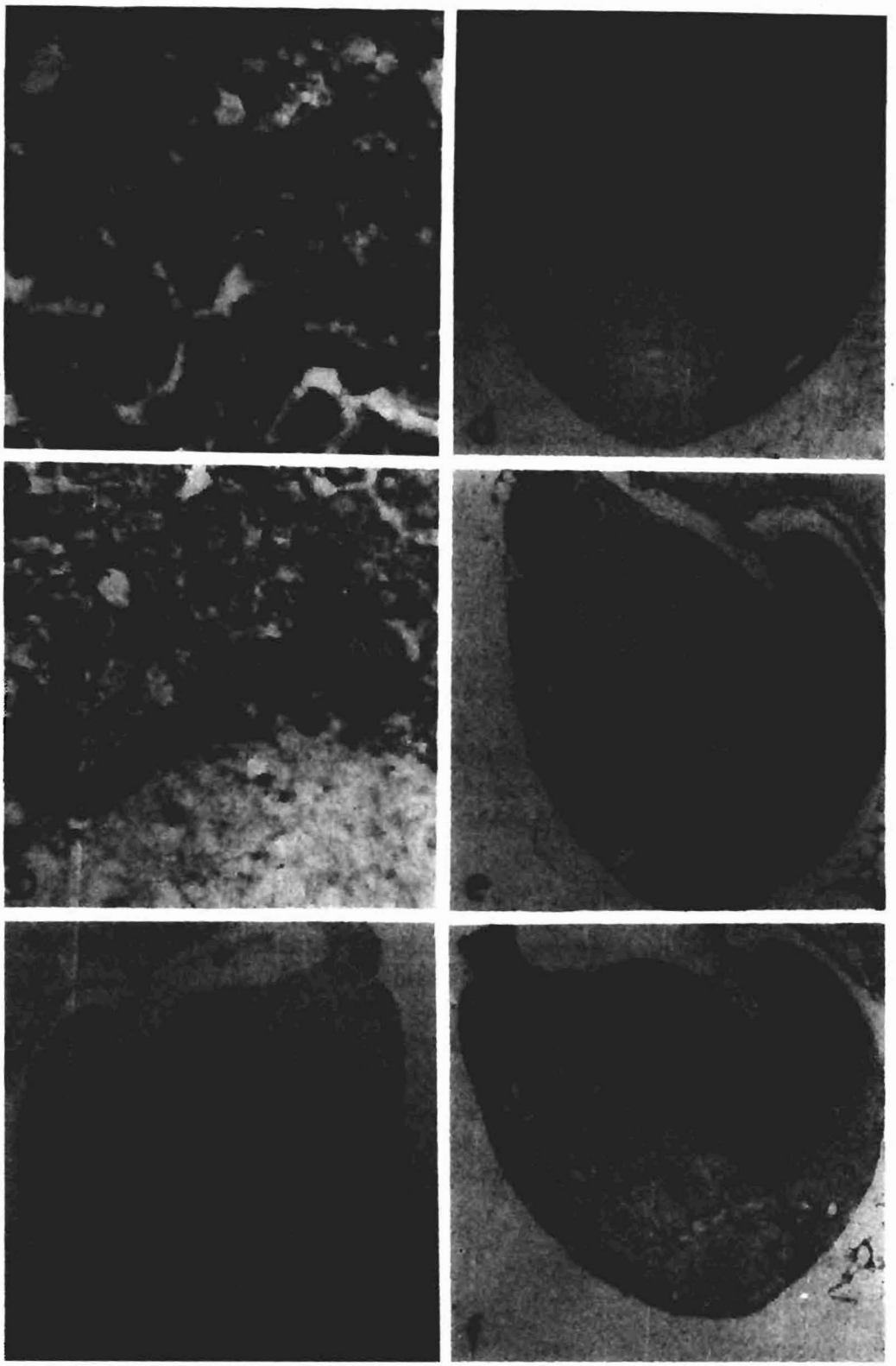
b- Female with prespawning ovary obtained from natural habitat. The SL cells are hypertrophied and with strong immunoreactivity.

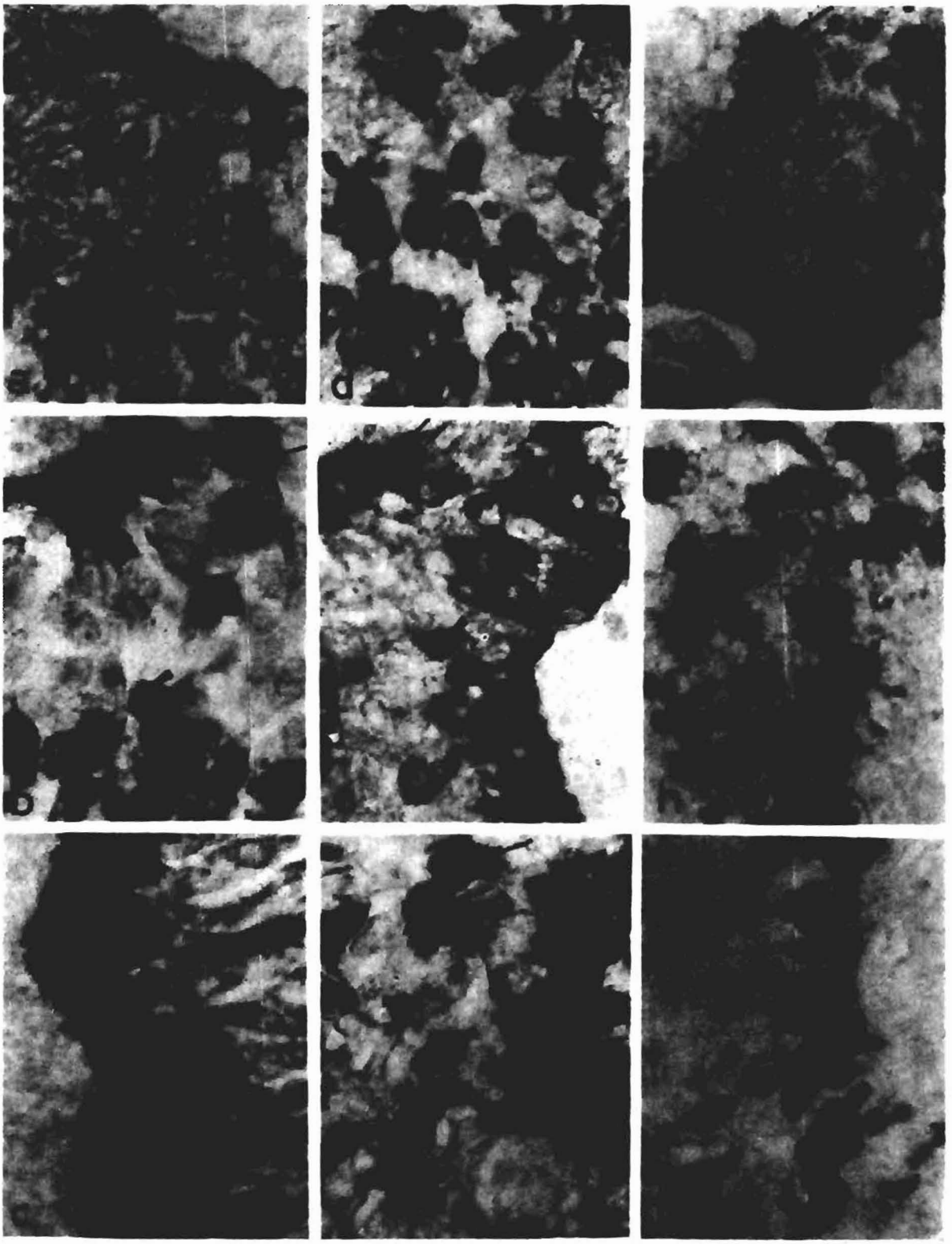
c- Ripe (spawning) female induced experimentally by injection of hormones. Note the increase of secretory activity as reflected by degranulation of most of SL cells (arrowheads). Also, some of granulated (arrows) SL cells are present.

d- Female with late-vitellogenic ovary obtained from captivity. The SL cells are increased in number and with strong immunoreactivity. Note some of SL cells are degranulated (arrowheads).

e- Female with atretic ovary obtained during the prespawning period from captivity. Note the weakly immunoreactivity of the SL cells.

f- Spent female obtained from natural habitat. The SL cells are decreased in size and number, and became degranulated.





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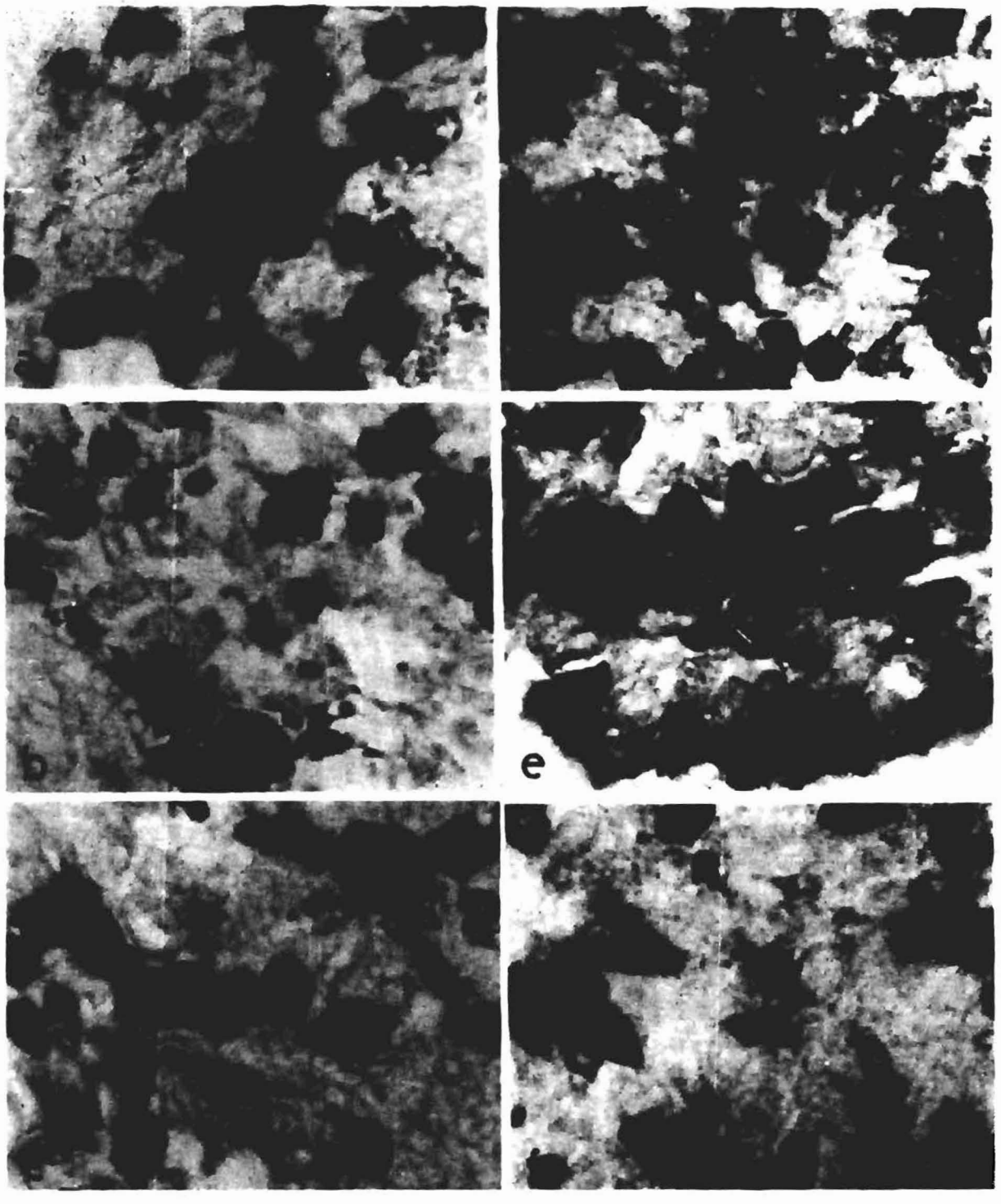

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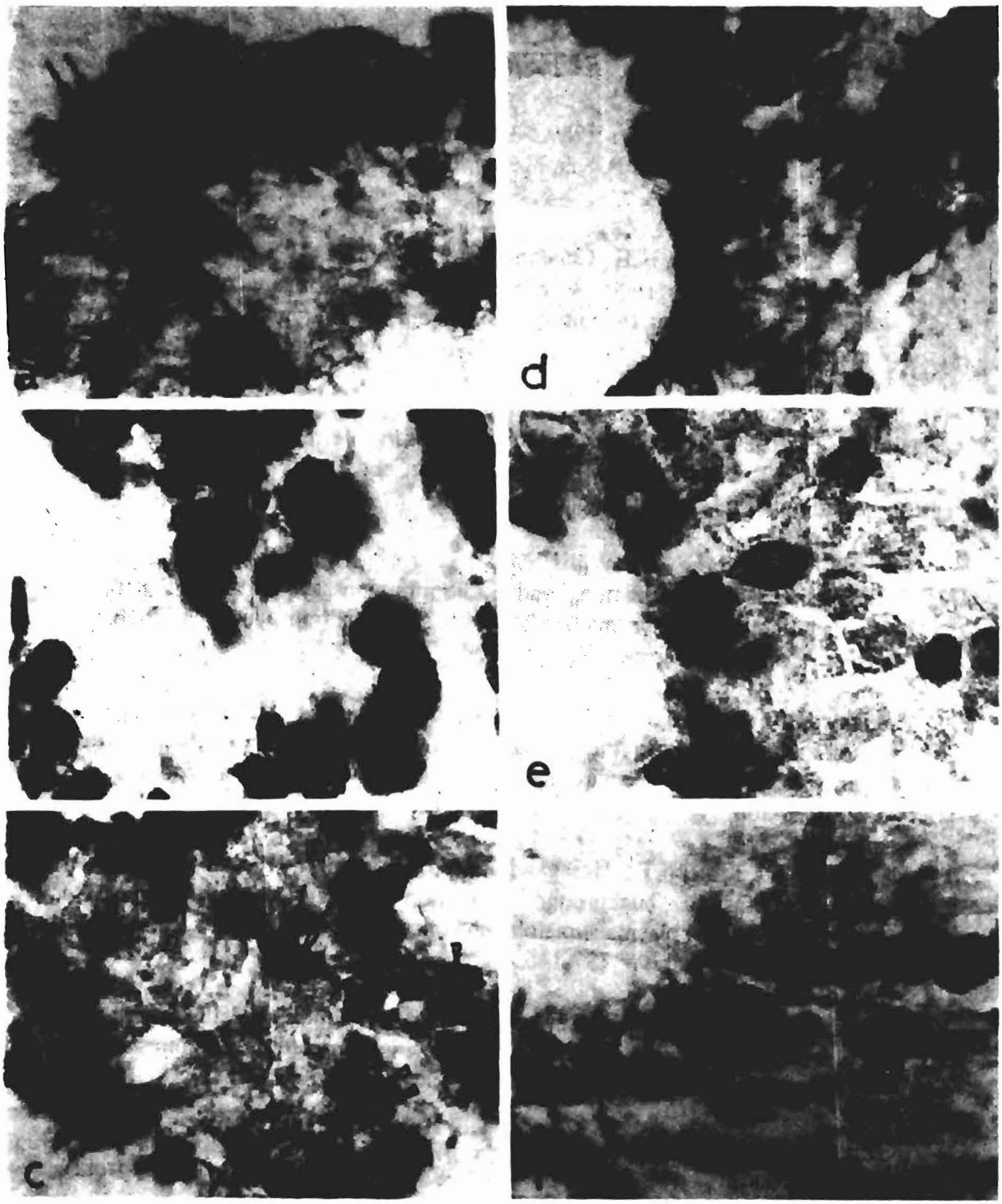

Fig.4

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