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Histochemical study of steroid synthesizing sites in a viviparous teleost, Zoarces viviparus

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The activities of Δ^5—3β-hydroxysteroid dehydrogenase (HSD), 11β-HSD and 17β HSD as well as pyridine nucleotide diaphorases (NADH-diaphorase and NADPH-diaphorase) were studied by histochemistry in the gonads, kidney and embryos of the eel-pout, *Zoarces viviparus* (L.), at various stages of the sexual cycle. NAD-linked 17βHSD (testosterone or oeastradiol-17β as substrate) was detected in the lobule boundary cells of the testis, and its activity was greatest at about the time of mating. On the lobule walls (interstitium) there may also be some cells/cell groups which show weak Δ^5-3β-HSD and 11β-HSD activities. The histochemical reaction of the 17β-HSD NADH-diaphorase system was greatly diminished by raising the incubation temperature from 22° to 37°C, but the diaphorase itself was intact.

In the ovary, despite the presence of diaphorases, HSD reactions could not be definitely demonstrated. However, the inter-renal tissue may be an important site for at least 3β-dehydrogenation both in adults and in embryos. Both inter-renal tissue and smaller oocytes in the ovary may also store steroids, probably in a conjugated form.

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1. Introduction

The reproductive cycle of the eel-pout, *Zoarces viviparus* (L.), has been found to be very regular in the Gulf of Finland (Kristoffersson & Pekkarinen 1975). The cycle includes an ovulation-spermiation period in July-August, pregnancy through the autumn and winter months until January-February, followed by gonadal growth and maturation. The growth of the following generation of oocytes is in progress as early as during pregnancy (Götting 1976, Pekkarinen 1980). The muscular tissue of the ovarian sac, which is responsible for expelling the embryos at birth, shows clear alteration in contractile activity and ability during different seasons (unpublished data of the authors).

How are these reproductive functions regulated? Öztan (1966) has found seasonal histological and histochemical alterations in the gonadotropic cells of the adenohypophysis of *Z. viviparus*. Korsgaard & Petersen (1979) have induced premature birth of embryos of the eel-pout with certain steroid hormones in late pregnancy. With regard to steroid metabolism, Kristoffersson et al. (1976) have traced metabolic pathways in the ovary of this species in vitro. However, there is no histochemical data about the steroidogenic sites of the eel-pout, although Bretschneider & Duyvené de Wit (1946) suppose that the postovulatory follicles in the ovary function as corpora lutea. The purpose of this study was to search for sites of steroid synthesis and to find possible seasonal changes in the activity of the key enzymes of steroid metabolism in this species.

2. Material and methods

Eel-pouts were caught with a bottom trawl and nets in the brackish water (salinity about 6 ‰) near Tvärminne Zoological Station, Gulf of Finland, at depths of 25—40 m. The fish weighed between 81.5 and 324.0 g, their lengths ranging from 28.5 to 42.0 cm. All fish were sexually mature.

After one week's rest in an aquarium with free-running natural brackish water (4—10°C) the fish were anaesthetized with neutralized MS-222^R (1:3000). Tissue samples for histochemical enzyme demonstrations were frozen fresh with liquid nitrogen and stored in liquid nitrogen before sectioning. Where it was necessary to check the stage of the sexual cycle, samples from the gonads were fixed in Bouin's fluid, dehydrated, and embedded in paraffin through butanol.

Paraffin sections were stained with Masson-Gomori (chromotrope — fast green; Gray 1954).

The sampling times and number of samples of ovaries were: April 19, 4; June 8, 2; June 26, 5; August 5, 3 (oocytes fully grown, non-ovulated); November 30 — December 8, 6 (pregnant); and January 5 — February 15, 8 (all but one pregnant). Many samples were taken from each ovary, especially in Nov — Dec, to include all possible steroidogenic sites. Testis samples were taken on April 19,2; June 8, 2; June 26, 4; August 5, 2 (breeding); December 15, 2; and January 25, 4.

The stucture of the kidney was viewed using Bouin-fixed paraffin sections made from different parts of the kidney stained in Masson — Gomori. Inter-renal tissue is found in the lobes of the anterior kidney, situated dorsolaterally from the oesophagus. For the enzyme demonstration, anterior kidneys (in January from 3 pregnant females, and in April from 3 females and 2 males) were removed with some muscle tissue of the neck and cut off caudally at the union of the lobes. Samples were also taken from the caudal end of the kidney where the corpuscles of Stannii are situated. The inter-renal tissue of embryos is situated in the same place as in adults. Whole embryos were frozen (in November and January), but most of the head and tail was discarded before sectioning.

Histochemical demonstration of hydroxysteroid dehydrogenases and diaphorases. Wattenberg's method according to Pearse (1972) was used as a basis to demonstrate Δ^5-3β-hydroxysteroid dehydrogenase (HSD), 11 β-HSD and 17 β-HSD. Dimethylformamide (Baker Chemicals) was used as a steroid solvent as recommended by Pearse. Substrates DHA (3β-hydroxy-5-androstene-17-one), hydrocortisone (11β, 17α, 21-trihydroxy-4-pregnene-3,20--dione) and testosterone (17β-hydroxy-4-androstene-3-one) were supplied by Sigma Chemicals Co., and pregnenolone (3β-hydroxy-5-pregnen-20-one), 11β-hydroxy-4-androsten-3,17-dione and oeastradil-17β (1,3,5(10)-estratriene-3,17β-diol) were supplied by Merck. NAD, NADP, NADH and NADPH were obtained from Beohringer, Mannheim. Nitro blue tetrazolium was from Sigma Chemicals Co. Polyvinylpyrrolidone (Kollidon K-25, BASF), at a final concentration of 7.5 % (Pearse 1972), was used in order to reduce diffusion artefacts in most incubation series. The pH of the incubation solution was usually 8.2, although 7.4 was also tested.

Sequential cryostat sections (12 μm) were collected on microscope slides. Incubation solution was dropped on the sections and they were incubated horizontally at 22° or 37°C for 1 h. A cold acetone bath before incubation and rinsing in 20 % ethanol were usually omitted, but their effects were checked occasionally with additional sections in the incubation series. Because the method depends on hydrogen carriers, endogenous diaphorases (NADH and NADPH tetrazolium reductases) were demonstrated in the tissue with NADH or NADPH as a substrate at pH 7.4. Each incubation series also included control sections with coenzymes (NAD or NADP) but with no additional substrate. Rat tissues were used as controls for the incubation solutions, which were prepared daily.

3. Results

Histochemical demonstration of the diaphorases and hydroxysteroid dehydrogenases in the testis. In winter and in spring NADH-diaphorase is found in spermatogenetic cysts and on the surfaces of the lobule walls, especially at the distal ends of the lobules. The lobule walls and the tunica of the testis are only lightly stained except for occasional spots of formazan. In summer, when the lobule boundary cells are prominent, NADH-diaphorase activity is strong in these cells (Fig. 1a). The strongest staining is seen at the distal ends of the lobules or somewhat inwards from them. Some staining is also seen in the residual cysts on the lobule walls.

Table 1. Diaphorase (NADHD and NADPHD) and 17β-HSD activities estimated in the testis of *Z. viviparus* during different seasons.

Date	N	17β-HSD(NAD)	NADHD	NADPHD
January 25	4	±	++++	+++
April 19	2	+(?)	++++	+++
June 8	2	++	++++	++/+++
June 26	4	+++/++++	++++/+++++	+++
August 5	2	+++/++++	+++++	+++/++++
December 15	2	+	++++	+++

± = no greater activity detectable than in the control sections
+ to +++++ = lowest detectable activity to the strongest staining

The distribution of NADPH-diaphorase is different from that of NADH-diaphorase. It occurs as spots in the tunica or lobule walls (Fig. 1b). The spots are more conspicuous in winter and are weaker and fewer in summer. In lobule boundary cells NADPH-diaphorase (Fig. 1c) is weaker than concomitant NADH-diaphorase.

Table 1 shows the diaphorase activities estimated from the staining intensity of the sections using the eye. 17β-hydroxysteroid dehydrogenase (17β-HSD) activity in the testes (testosterone or oestradiol-17β as substrate and NAD as coenzyme) is estimated using a low magnification (x100). The widely accepted ranking system (+ – +++++) was used. 17β-HSD occurs especially in the lobule boundary cells and its activity is thus seen to be greatest in summer when these cells are hypertrophied (Fig. 1d). Lobule walls are also lightly stained. Some net formazan is perhaps also formed in December at the surfaces of the lobule walls and in April weakly in the spermatogenetic cysts on the lobule walls. Residual cysts in summer also show weak staining.

The activities of Δ^5-3 β-HSD and 11 β-HSD were not quite distinct. However, it seems that these (with NAD or NADP as coenzyme) occur occasionally as spots in the lobule walls and/or in the tunica (Figs 1e and 1f). No clear seasonal rhythm could be seen in their occurrence. The border of the spots was not exact, as if some diffusion had occurred. These spots may represent the spots mentioned above in connection with NADHD and NADPHD. The cells could not be identified in stained paraffin sections. Cells in the tunica and lobule walls are flattened and do not look like active Leydig cells of a mammalian testis. The evaluation of weak enzyme activities was a little difficult because of some formazan formation in the control sections, too.

When the incubation was carried out at 37°C,

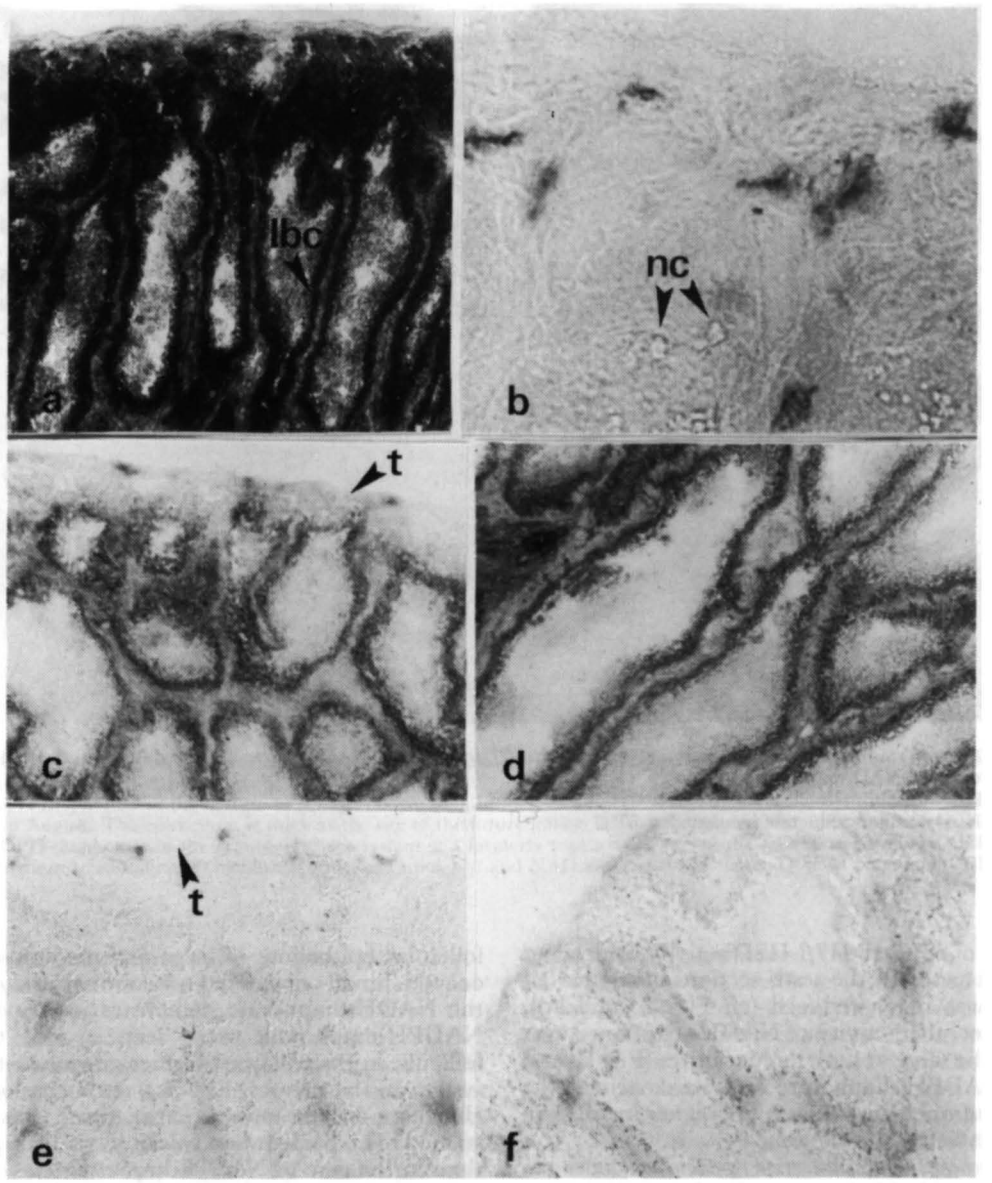

Fig. 1. Results of the histochemical enzyme demonstrations in the testis of *Zoarces viviparus*.
a) NADH-diaphorase in a testis in August (+++++). Note the very strong zone of diaphorase near the distal ends of the lobules. lbc = lobule boundary cells. x110.
b) NADPH-diaphorase spots in the margin of a testis in December. nc = necrotic cells in the lobules (common in winter, Kristoffersson & Pekkarinen 1975). x530.
c) NADPH-diaphorase in the same testis as in a) (August, +++). t = tunica. x110.
d) 17β-HSD activity (testosterone as substrate and NAD as cofactor) in the lobule boundary cells of the same testis as in a) and c) (+++/++++). x110.
e) Formazan spots in the tunica and in the lobule walls of a testis incubated with DHA and NAD in January. x110.
f) Spots in the lobule walls of a testis incubated with 11β-hydroxyandrostenedione and NAD in August. x110.

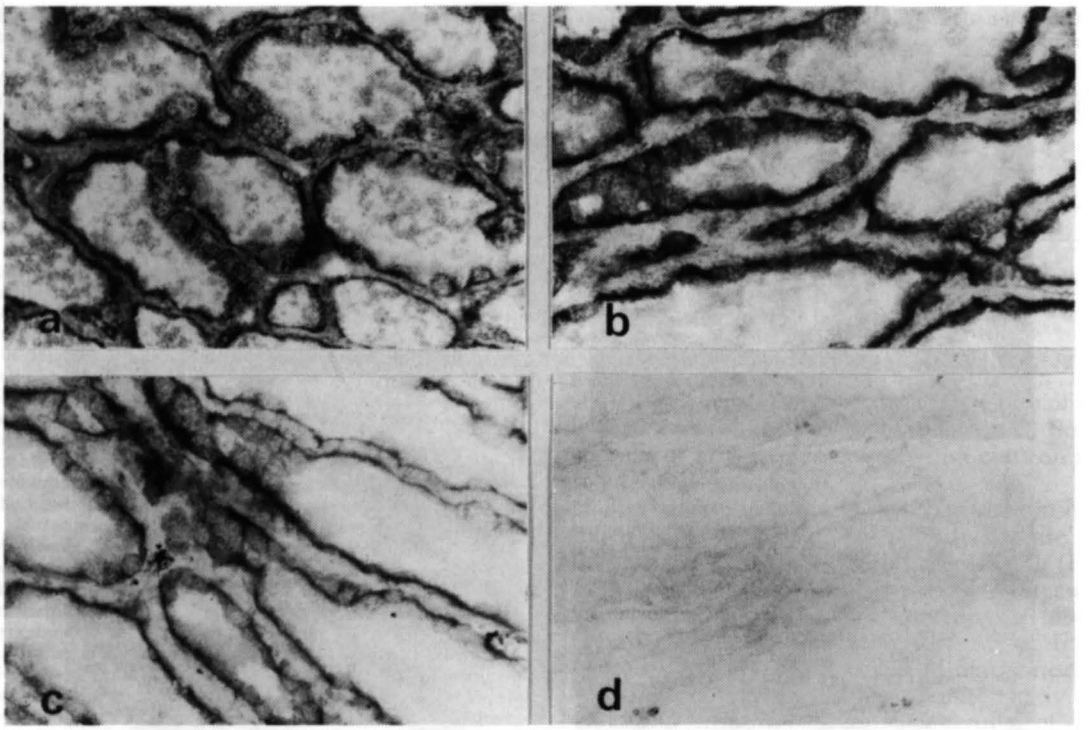

Fig 2. Effect of raising the incubation temperature from 22° to 37°C on NADH tetrazolium redustace and 17β-HSD (testosterone and NAD) in a testis on June 26.
a) NADH tetrazolium reductase incubated at 22°C
b) NADH tetrazolium reductase incubated at 37°C
c) 17β-HSD incubated at 22°C
d) 17β-HSD incubated at 37°C. x85.

instead of 22°C, the 17β-HSD activity, which had been detected in the testis sections incubated at 22°C, was much reduced (cf. Figs 2c and 2d). However, the activity of NADH-diaphorase was about the same at both temperatures (Figs 2a and 2b). NADPH-diaphorase was weakened at the higher temperature. Later, the higher incubation temperature was abandoned.

Histochemical demonstration of the diaphorases and hydroxysteroid dehydrogenases in the ovarian tissue. NADH-diaphorase activity was strong in small and medium-sized oocytes and at the margin of large oocytes, in the follicle epithelium (granulosa) and in the epithelium surrounding the ovarian inner surface with its villi or calyces (Figs 3a and 3b). Sometimes small spots were encountered in the theca folliculi and in postovulatory follicles (collapsed former granulosa and theca) in the calyces nutriciae. Otherwise there was a moderate background colour in the muscle tissue and in the stroma. Scar tissue and inflammatory tissue stained a little darker than the background. NADPH-diaphorase was very active in the follicular epithelium of large and medium-sized oocytes. Small oocytes did not stain as darkly as in the NADH-diaphorase demonstration. Spots of NADPH-diaphorase were seen in the theca folliculi, in the collapsed former granulosa-theca system in the calyces (Fig. 3c), and occasionally elsewhere in the stroma. Scar tissue was also stained. The background colour was a little paler than in the case of NADH-diaphorase.

In hydroxysteroid dehydrogenase demonstrations small and medium-sized oocytes stained bluish (Fig. 3d). A light blue colour was also often seen in the epithelium, sometimes in the atretic follicles, in the granulosa-theca system of former follicles (as spots) and rarely in the theca of growing follicles. We could find neither any clear substrate preference in the colour formation, nor clearly indicated net staining which would have exceeded the colour in the control sections. Furthermore, we could see no outstanding changes in the enzyme reactions in different seasons.

Histochemical demonstration of the diaphorases and

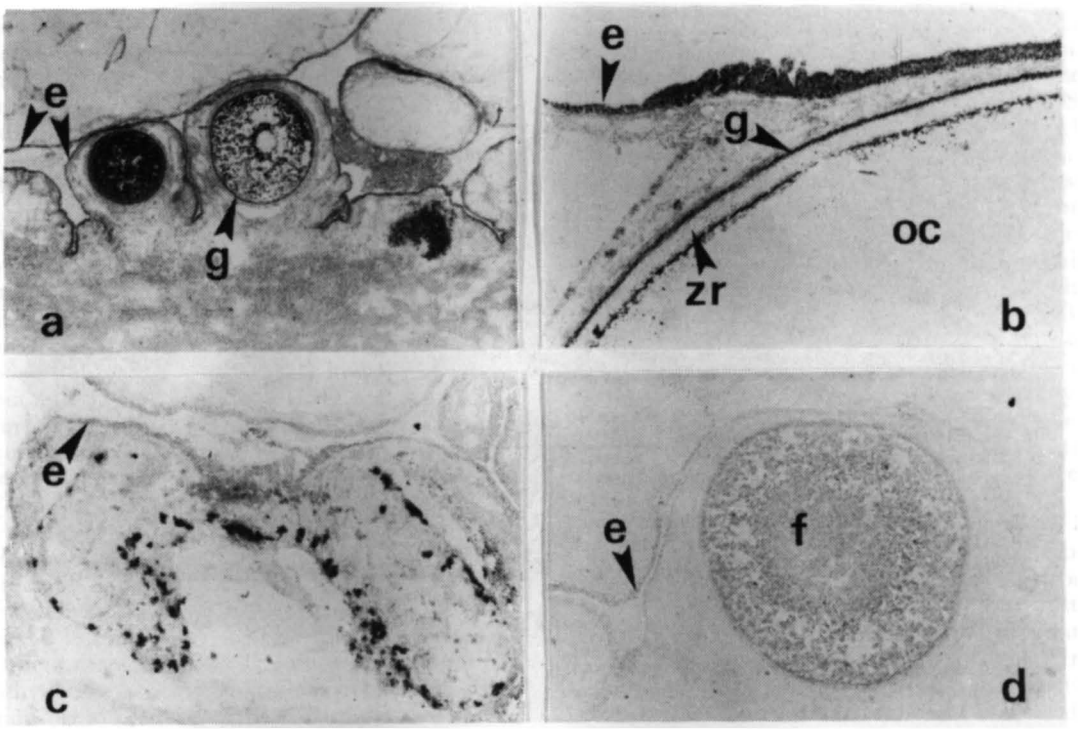

Fig 3. Histochemical results from the ovary of *Zoarces viviparus*. a) NADH-diaphorase in an ovary in August. g =granulosa, e = inside epithelium of the ovary. x34.
b) NADH-diaphorase in the margin of a large oocyte (oc), in its follicular epithelium (g) and in the inside epithelium of the ovary in August. The epithelium is thick at the site of the future follicle rupture (ovulation site). zr = zona radiata. x85.
c) NADPH-diaphorase in the granulosa-theca system of a formerly ruptured follicle in the same ovary as in b). x85.
d) A medium-sized follicle (f) incubated with oestradiol-17β and NAD in August (see the text). x85.

hydroxysteroid dehydrogenases in the kidney. Diaphorase reactions were strong in the inter-renal tissue and in the renal tubules (apart from the renal corpuscles), but weaker elsewhere in the kidney, including the corpuscles of Stannii. The arrangement of inter-renal cells as groups or glomeruli is seen in Fig. 4a. Intermingled with the inter-renal tissue there were some groups of cells which stained only lightly in the diaphorase demonstration and often the margin of the inter-renal tissue was darkest. In some specimens inter-renal cells seemed to be as overloaded with formazan.

Incubation of the inter-renal tissue with 3β-substrates gave strong staining (Fig. 4b). It seemed to be slightly more intense with coenzyme NADP than with NAD and slightly less intense with pregnenolone than with DHA. Again there were som lightly stained cell groups, as in the diaphorase demonstration. However, the inter-renal tissue stained darkly in control sections, too (especially with NADP), even when the incubation was carried out after acetone extraction. Nevertheless, the staining in the interrenal tissue incubated with 3β-hydroxysteroids seemed to exceed the control staining, and that seen in the sections incubated with the other substrates. The renal tubules were moderately stained in both the control sections and in the HSD demonstrations, but the corpuscles of Stannii were hardly stained at all. No seasonal or intersexual differences could be judged from these few preparations.

The embryonal kidney showed enzyme reactions similar to those of the kidney in adults, except that the intensity of formazan was weaker. In the older embryos (January, late pregnancy) Δ^5-3β-hydroxysteroid dehydrogenase activity was somewhat stronger than in the younger embryos (November, mid-pregnancy) (Figs 4c and 4d).

4. Discussion

In fish testis there may be at least two possible

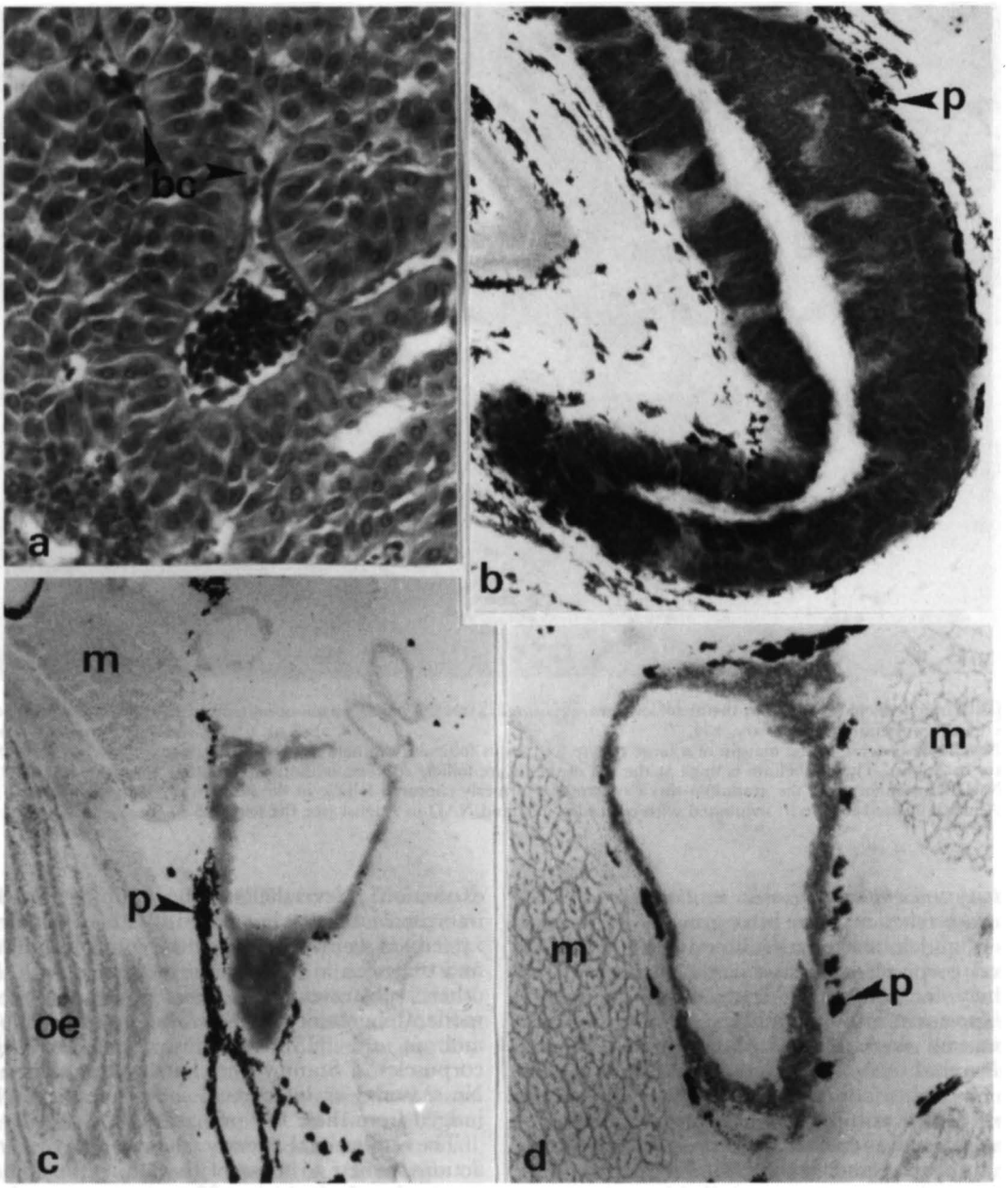

Fig 4. Structure and Δ^5-3β-HSD reactions in the inter-renal tissues of adult *Zoarces viviparus* and embryos.
a) Inter-renal tissue of an adult in February stained with Masson — Gomori (paraffin section). bc = blood capillary. x370.
b) Inter-renal tissue incubated with DHA and NAD in April. p = pigment. x58.
c) Inter-renal tissue of an embryo on November 30, incubated with DHA and NAD (anterior upwards). oe = oesophagus, m = muscle tissue. x58.
d) Inter-renal tissue of an embryo incubated with DHA and NAD on January 8. x125.

steroidogenic structures: interstitial cells (Leydig cells) and lobule boundary cells. In *Fundulus heteroclitus* HSD activity was seen mostly in the interstitial cells (Bara 1969). O'Halloran & Idler (1970) found weak 3β-HSD activity in the lobule boundary cells in the testis of *Salmo salar*. Both cell types appear in the testis of *Cymatogaster aggregata* and they show Δ^5-3β-HSD activity (Wiebe 1969). Our results suggest that in the testis of *Zoarces viviparus* there may also be two different structures involved in steroid metabolism. At about the time of mating there was strong 17β-HSD activity in the hypertrophied lobule boundary cells. These cells are invisible in winter, but the site of weak 17β-HSD reaction on the surfaces of the lobules in December may represent quiescent lobule boundary cells. Although interstitial Leydig cells have not been identified in *Zoarces* testis, some cells/cell groups in the lobule walls seemed to contain Δ^5-3β-HSD or 11β-HSD. However, steroidogenic function in the spots observed in the tunica is questionable. These cells may be of some other active cell type, in which NADPH(NADH) - diaphorase is also strong. Other reactions may confuse hydroxysteroid dehydrogenase demonstrations in histochemistry (nonspecific alcohol dehydrogenase and 'nothing dehydrogenase', Pearse 1972). The HSD methods include many sources of error (Høyer & Andersen 1970), and they must be further developed.

The temperature of incubation must be carefully taken into account when studying enzymes of heterothermic animals. The temperature lability of the HSD-diaphorase enzyme system in *Z. viviparus* was not dependent on NADH-diaphorase because NADH tetrazolium reductase was not affected by raising the temperature from 22 to 37°C. NADPH-diaphorase seemed to be more labile. Kime & Hews (1978) criticize authors who have used a temperature of 37°C in biochemical steroid incubations of tissues of the northern pike because 11-oxygenating enxyme diminishes rapidly above 26°C in species such as the trout (Kime 1978a), Suzuki & Tamaoki 1972). The optimum temperatures of enzymes may vary very much, depending on the species and even depending on the enzyme in question. In trout testis, raising the incubation temperature from 16° to 37° did not enhance, but rather depressed, 11β-hydroxylation while 17β-HSD activity was increased more than twice as much as the activity at 16° (Suzuki & Tamaoki 1972). NADPH-diaphorase is probably connected with steroid hydroxylations.

At least four structures in the fish ovary have been suggested as possible steroid transforming sites. They are granulosa cells, interstitial cells (special theca cells?), postovulatory follicles (Lambert 1978) and atretic follicles (e.g., Saidapur & Nadkarni 1976). Thus the most probable steroidogenic sites are follicles or their derivatives. It was surprising to find that HSD reactions in the ovary of *Z. viviparus* could not be convincingly demonstrated by histochemistry. It has, however, been shown that the ovarian tissue of the eel-pout metabolizes steroids, e.g. pregnenolone and 17α-hydroxyprogesterone (Kristoffersson et al. 1976). Similarly, the testicular tissue of pike converted several substrates to other steroids, but the histochemical reactions for 3β-HSD and 17βHSD were negative (Lupo di Prisco et al. 1970). The authors attributed the negativity of the histochemical reactions to poor sensitivity of the methods. Gresik et al. (1973) found that Leydig cells of *Oryzias latipes* showed characteristics of steroidogenic cells but the HSD reactions were negative.

The inter-renal tissue of *Z. viviparus* must be regarded as an important candidate as a steroid synthesizing site. However, because of strong control staining, the specificity of our histochemical reactions for different steroid substrates was poor. Strong diaphorases and strong control staining indicated, at least, that the tissue could be metabolically very active. Perhaps it contains large amounts of endogenous steroids or other substrates for dehydrogenases. It is odd that the substrates, if they were steroids, were not extracted by acetone. Also small and medium-sized oocytes in the ovary contained some non-acetone-extractable hydrogen donor. It is known that the gonads of non-mammalian vertebrates glucuronidate and sulphurylate steroids in larger amounts than in mammals (e.g. for fishes: Colombo et al. 1972, Hews & Kime 1978, Kime & Hews 1978, Kime 1978b). At least hydroxysteroid sulphates are known to give histochemical staining in the presence of hydroxysteroid dehydrogenase (Baillie et al. 1966).

There was some evidence for an initial step of the steroid synthesizing pathway, 3β-dehydrogenation, in the inter-renal tissue. In biochemical incubations the ovarian tissue converted pregnenolone to progesterone in only minimal amounts, and less than 30 % of progesterone was metabolized (Kristoffersson et al. 1976). Korsgaard & Petersen (1979) have been able to provoke the birth of embryos of the eel-pout by treatment with progesterone or combined progesterone and oestradiol in late pregnancy. But it is not confirmed that progesterone acts as a physiologically active hormone in the eel-pout. At least its function would differ from the classical function in mammals and its time of action could be short. Korsgaard & Petersen could not, however, abort the embryos during early

pregnancy. There must therefore be some other regulating factors. On the basis of the histochemical staining the inter-renal tissues are functional even in midpregnancy embryos of the eel-pout. Kujala (1978) suggests that in guppies embryonic development may serve as an internal clock and embryonic corticosteroids may trigger follicular processes, ultimately resulting in parturition. It must be noted that the embryos of the guppy develop within the follicle and their parturition may be equivalent to egg release in oviparous fishes. Whether embryonal hormones reach maternal tissues and have an effect on them in *Zoarces viviparus* is not known.

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