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Changing Enzyme Activities in Maturing Gonads of the Winter Flounder, Pseudopleuronectes americanus

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INTRODUCTION

In our laboratory, the winter flounder *(Pseudopleuronectes americanus)* is a frequently used test animal during the winter months, when it is easily obtainable from the waters of Long Island Sound; these months are also the animal's spawning period. During our studies of sublethal heavy-metal effects in various tissues of this fish, we found widely disparate enzyme activities in gonads at different stages of maturation, for both sexes. It seemed pertinent, therefore, to undertake a study of normal metabolic patterns in developing gonads of male and female winter flounder, the better to interpret experimental data for each developmental stage.

The enzymes examined were those we have studied in our heavy-metal experimentation: metalloenzymes and metal-modulated enzymes, whose properties can be altered by metal challenge; and representative glycolytic and pentose shunt enzymes, whose induction under sublethal stress signals increased rates of energy mobilization and reductive biosyntheses, respectively. Of the latter group, only one does not fall into the first group, as well.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49; G6PDH), the pacemaker pentose shunt enzyme, is a commonly used indicator for the activity of that pathway, which in turn indicates the rate of reductive biosynthetic activity, as in lipogenesis and nucleic acid production. Rates are high in rapidly metabolizing tissues like hepatomas (Weber, 1963; Gumaa *et al.* 1968) and newly-fertilized sea urchin eggs (Krahl, 1956; Bäckström, 1959), for example, and during intermolt in crustaceans (McWhinnie and Corkill, 1964) and cold acclimation (Kanungo and Prosser, 1959; Hochachka and Hayes, 1962) and stress (MacInnes *et al.*, 1977) in fish. G6PDH is also modulated by magnesium ion, a positive effector. Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40; PK) and glucosephosphate isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9; GPI) were selected to monitor glycolysis. PK is another Mg^{2+}-modulated enzyme, and also requires K+ or similar monovalent cation for activity (Boyer, 1962), whereas GPI has no known metal requirement, but is inhibited by high concentrations of Mg^{2+} and other metal cations (Topper, 1962).

Of the metalloenzymes studied, all of which incorporate Zn in their structure, two are glycolytic enzymes involved in maintaining a proper NADH:NAD balance in the cell: lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27; LDH) and malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37; MDH). Relative activities of these two enzymes have been used to indicate shifts between aerobic

and anaerobic metabolism in marine invertebrates (Hochachka and Somero, 1973; Livingstone, 1976) low-salinity stress in a marine worm (Cripps and Reish, 1973) and decapod crustacean (Gould, unpublished observations). The other two Zn enzymes are very active in the male gonad: acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2; APh) and carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1; CA). Both are associated with the mature but not the immature tissue (Gutman and Gutman, 1938; Hodgen *et al.*, 1969)[1].

METHODS AND MATERIALS

Animal Collection

The fish were taken by otter trawl during the months of March and April from the estuarine waters of the Thames River, Connecticut, and from the waters of Long Island Sound outside of Milford Harbor.

Rating of Gonad Maturation

Gonads were excised and rated by gross observation for degree of maturation, then sealed in air-tight plastic pouches and held frozen (-80°) until analysis, no longer than 3 weeks. Maturation stages were rated R_0 through R_4, beginning with the very small, very immature gonads (R_0), up to the swollen, ripest stage (R_4). PS designated partially spent gonads, and S, the completely spent, flaccid tissue.

Tissue Preparation

Gonad tissues were prepared individually, except for the immature, very smallest specimens, which were pooled. The whole organs of small gonads were used in making the tissue preparations, whereas only a portion of the much larger R_3, R_4, and spent organs were used. Accordingly, R_3 and especially R_4 preparations were almost entirely gametic and R_0 somatic, with proportionately larger gametic fractions in R_1 and R_2. Spent tissue consisted primarily of thickened gonad walls.

The homogenates were 1:4, w/v, in cold 0.88 M sucrose containing 1 mM dithiothreitol, and were ground in glass homogenizers with 25-µm glass powder. Homogenates of male gonad tissues were frozen (-29°) for 1-3 days, then thawed, sonicated (3 10-second bursts, setting 4, Branson Sonifier W-140[2]), and centrifuged, as this procedure yielded preparations of higher activity than was the case for untreated preparations. Centrifugation was at 40,000 *g* and 4° for 45 min, producing clear supernatants (5xM, 5xF preparations). Further dilutions to 10x were made with distilled water.

Enzyme Assays

All solutions were made with doubly glass-distilled water. Biochemicals were obtained from Sigma Chemical Company, St. Louis, Mo. Optimal assay protocols were worked out with mature gonads (R_3 and R_4), and the same protocols were used for every maturation stage. Assay temperature was 25°C. Activities were calculated

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[1] *Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; NADP, nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; G6P, glucose-6-phospate, disodium salt; TEA, triethanolamine; ADP, adenosine 5'-diphosphate; PEP, phospho(enol)pyruvate, tricyclohexylamine salt; EDTA, ethylenediamine tetraacetate, disodium salt, dihydrate; cis-OA, cis-oxaloacetic acid; HEPES, (N-2-hydroxyethyl)-piperazine (N'-2-ethane)-sulfonic acid; F6P, fructose-6-phosphate, potassium salt.*

[2] *Reference to trade names does not constitute endorsement by the NMFS, NOAA.*

from the fastest portion of the curve 25 seconds after start of reaction. Units of activity were either conversion of 1 µmole substrate/min/mg biuret protein (for pyridine nucleotide-linked enzymes), or absorbance change of 0.001/min/mg biuret protein. Assay concentrations (mM) in 3.00-ml reaction volume were: *G6PDH:* Tris buffer pH 8.0, 80; NADP 0.3; G6P, 0.5; $MgCl_2$, 2.5; 0.10 ml 5xM/0.20 ml 5xF; *PK* (based on Bücher and Pfleiderer, 1955): TEA buffer pH 7.5, 60; $MgCl_2$, 8.3; KCl, 75; NADH, 0.15; ADP, 0.32; PEP, 0.53; 175 units LDH; 0.10 ml 5xM/0.10 ml 10xF; *GPI:* Tris buffer pH 8.0, 80; NADP, 0.3; $MgCl_2$, 2.5; F6P, 0.67; 4 units G6PDH; 0.10 ml 5xM/0.10 ml 10xF; *MDH* (based on Grimm and Doherty, 1961): glycine-EDTA buffer pH 9.0, 90 glycine and 0.9 EDTA; NADH, 0.15; cis-OA, 1.0; 0.10 ml 10xM and F; *LDH* phosphate buffer pH 7.5, 88; NADH, 0.15; NA pyruvate, 10; 0.10 ml 5xM/0.20 ml 5xF; *CA* (based on Pocker and Stone, 1967): K_2HPO_4 pH 8.84, 12.7; *p*-nitrophenyl acetate (solubilized in acetone to final 3% soln in water), 10; inhibitor acetazolamide, 0.04; 0.10 ml 5xM; APh: acetate buffer pH 5.0, 100; *p*-nitrophenyl phosphate, 4.2; 0.10 ml 5xM.

RESULTS AND DISCUSSION

Increased pentose shunt activity, as measured by G6PDH, was seen in the ripe R_3 and R_4 gonad stages in both male and female winter flounder (Fig. 1a). In the serum of female flounder *(Platichthys flesus)* with developing ovaries and in estradiol-treated fish, Emmersen and Petersen (1976) observed increased amounts of the yolk-precursor lipophosphoprotein vitellogenin, whose subsequent metabolism in the ovary would require the reducing equivalents produced by the pentose shunt. The apparently smaller increase in the maturing female gonads is probably due to our using mg protein in calculating activity; the greater amount of inert protein in maturing oocytes contributes to a misleadingly low calculated activity. In spent tissue of both sexes, G6PDH values dropped below the rates for immature tissue, indicating very low biosynthetic activity.

It is well to remember the disproportionate amount of gametic tissue in the preparations of mature gonads, and the somatic nature of the immature and spent gonads. Enzyme activity that increased with gonad maturation, therefore, is probably associated with gametes, and activity that decreased, with somatic tissue.

The reverse picture was seen in PK, reflecting overall glycolysis. Activity dropped sharply in maturing male gonads, and rose again as sharply in the spent tissue (Fig. 1b). GPI data followed the same course, to a less marked degree. In the female gonad, PK increased up to the R_2 stage, after which its metabolic pattern was similar to that for the male. The brief increase might be construed as a greater demand for energy in the initial stages of gametogenesis in the female. But here gametogenesis presents a complex picture: the ripening ovary of the female winter flounder has been found to contain maturing oocytes as well as atretic and immature ones (Dunn and Tyler, 1969), a situation not conducive to clear-cut biochemical profiles. Moreover, GPI showed little variation throughout maturation of the female gonad and even in spent tissue.

During gonad development, the activity ratio PK/G6PDH decreased in both sexes as the carbon flux shifted to favor the pentose shunt over the Embden-Meyerhof glycolytic pathway (Table 1). Although overall PK values in the female gonad were higher than in the male and the overall G6PDH lower, the ratios were generally similar for both sexes, except for markedly higher values in the spent female gonad. Values for R_0 through R_2 overlapped sufficiently in the female to be pooled (note range, Table 1), as was also true for R_3 and R_4. The high values through R_2 in the female again signaled rapid glycolysis and low shunt activity, which was also the case in spent tissue.

Patterns for both LDH and MDH were different for the two sexes. LDH activity was negligible in the female throughout gonad maturation, rising slightly only in spent tissue (Fig. 1d). Conversely, LDH in the immature male gonad was relatively high,

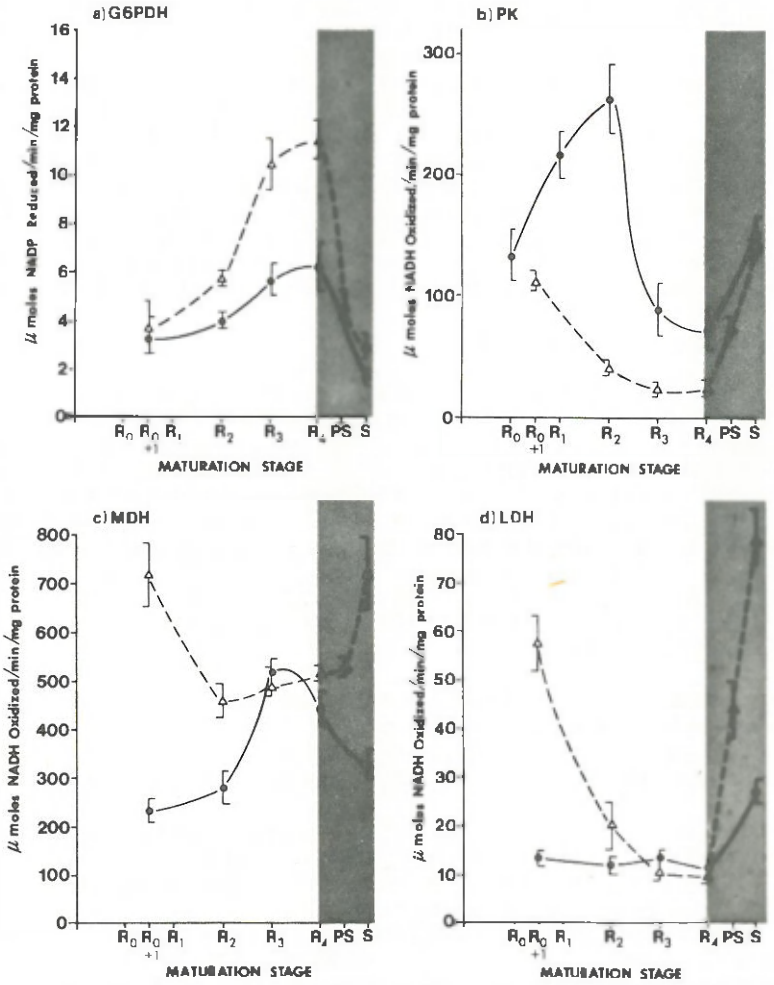

Fig. 1. Activities of (a) glucose-6-phosphate dehydrogenase, (b) pyruvate kinase, (c) malate dehydrogenase; and (d) lactate dehydrogenase in immature (R_0-R_2), maturing (R_3 and R_4), and spent (PS and S) gonads of male (- -Δ- -) and female (——o——) winter flounder. The shaded area to the right in each figure represents the spent stages, and vertical lines for each point, standard error.

declining to the same low levels as in the female at R_3 and R_4 stages, then increasing sharply in spent tissue, following the course of glycolysis (Fig. 1b).

To a lesser extent, MDH activity in the male gonad (Fig. 1c) also followed the glycolytic pattern. The female MDH pattern was the reverse, starting low in the immature, rising at R_3 and R_4 to approximately the same levels as in the male at that stage, then falling off again in the spent tissue; here the general picture resembled that of the pentose shunt (Fig. 1a).

TABLE 1. Comparison of Glycolytic and HMP Shunt Activity (PK/G6PDH) in Maturing and Spent Winter Flounder Gonads

	Male				Female			
Stage	n	$\bar{x}$	*S.E.*	*(Range)*	n	$\bar{x}$	*S.E.*	*(Range)*
$R_o + R_1$	6	51.4	± 8.6	(27.7-88.8)	5[a]	53.1	± 3.8	(45.7-66.1)
R_2	9	7.0	0.9	(3.7-10.4)				
R_3	15	2.6	0.3	(1.0- 4.9)	7[b]	6.3	1.0	(3.6-10.2)
R_4	18	2.4	0.3	(1.0- 5.6)				
PS	12	24.2	4.9	(4.1-56.2)				
S	4	49.7	6.0	(36.5-64.7)	3	90.1	9.0	(78.9-108.0)

a includes R_2 data; b includes R_4 data.

Despite the contrasting MDH patterns, however, MDH/LDH ratios followed similar trends (Table 2), suggesting a common mechanism for redox regulation at analogous stages of development in the two sexes.

The sperm-specific LDHX (Goldberg, 1963; Blanco and Zinkham, 1963), found in some mammals (Baldwin and Temple-Smith, 1973) and birds (Zinkham and Isensee, 1969), apparently is not present in this fish. Its presence has been correlated with the process of spermatogenesis (Hawtrey and Goldberg, 1968), whereas overall LDH activity decreased in the maturing flounder testis. The gonadal LDH seen here is probably the muscle isoenzyme, LDH_A; Markert and Holmes (1969) found the distribution of LDH isoenzymes in pleuronectid tissues to be highly skewed toward that form, unlike most other teleosts. Their extensive study, however, did not include gonads.

TABLE 2. Comparison of activities, MDH/LDH, in Maturing and Spent Winter Flounder Gonads

	Male				Female			
Stage	n	$\bar{x}$	*S.E.*	*(Range)*	n	$\bar{x}$	*S.E.*	(Range)
$R_o + R_1$	4	12.2	± 2.5	(7.0-18.8)	8	19.8	± 2.4	(8.0-29.2)
R_2	6	26.2	3.3	(14.4-35.2)	8	24.4	1.8	(14.6-29.9)
R_3	11	47.9	6.0	(22.0-78.6)	8	34.5	2.0	(30.0-46.1)
R_4	7	51.2	4.8	(33.3-70.3)	8	42.2	3.9	(31.3-59.0)
PS	11	15.1	2.2	(6.6-28.1)				
S	4	9.3	1.0	(8.3-12.2)	8	12.3	1.3	(7.4-15.9)

TABLE 3. Length and Weight of Winter Flounder Used in This Report

Gonad Stage		n	Length (cm) $\bar{x}$	S.E.	(Range)	Weight (g) $\bar{x}$	SE	(Range)
R_o	F	12	12.8 ±	1.0	(8.7-17.5)	25.2 ±	5.2	(7-56)
	M	19	19.0	0.9	(9.9-23.8)	75.9	8.2	(11-148)
R_1	F	15	21.8	0.7	(16.3-24.5)	102	8.5	(41-158)
	M	5	24.5	1.6	(19.2-29.0)	126	16.3	(72-170)
R_2	F	11	24.8	1.1	(18.0-32.3)	177	25.6	(66-370)
	M	9	21.0	1.9	(12.8-30.4)	102	24.3	(19-237)
R_3	F	9	27.3	1.1	(22.0-32.0)	237	27.6	(115-367)
	M	17	20.4	1.2	(15.3-32.1)	105	23.2	(23-408)
R_4	F	9	30.9	1.6	(24.5-39.4)	432	71.1	(135-748)
	M	18	24.5	0.9	(19.1-33.2)	177	24.1	(71-451)
PS	M	12	20.0	0.6	(17.6-24.6)	82	8.0	(59-149)
S	F	12	28.7	1.3	(19.0-34.2)	277	36.2	(129-557)
	M	5	21.2	0.5	(19.7-22.4)	92	8.7	(67-116)

TABE 4. Carbonic Anhydrase (CA) and Acid Phosphatase (APh) in Maturing and Spent Male Gonads of Winter Flounder

	Stage	n	$\bar{x}$		S.E.	(Range)
CA:	$R_o + R_1$	8	9	±	4	(0- 28)
	R_2	11	136		23	(0-206)
	R_3	17	250		15	(125-350)
	R_4	18	250		15	(91-348)
	PS	12	92		16	(15-185)
	S	4	15		5	(0- 23)
APh:	$R_o + R_1$	6	39		15	(9-110)
	R_2	7	70		8	(44- 97)
	R_3	12	99		7	(58-149)
	R_4	14	109		4	(90-133)
	PS	7	33		4	(14- 47)
	S	1	34			

Unit of activity: change in absorbance of 0.001/min/mg biuret protein at 400 nm.

The widely disparate MDH patterns might be construed as reflecting different proportions of isoenzymes. In a study of the cytoplasmic (sMDH) and mitochondrial (mMDH) forms, Kitto *et al.*)1967) found both in supernatant fractions, "probably because of some mitochondrial breakage" during homogenization; mMDH is not firmly bound to the mitochondrial membrane system (Rendon and Waksman, 1971). Moreover, increased mitochondrial fragility has been observed in fasted animals (Scrutton and Utter, 1968), and the winter flounder fasts during its spawning season. Because MDH in the male follows the glycolytic pattern, a reasonable inference is that the predominant isoenzyme is the Krebs cycle-associated form, mMDH. The same line of thought would lead one to suspect that because MDH in the female follows the pentose shunt pattern, the predominant isoenzyme might be sMDH, which is poised toward the generation of reducing equivalents. A study of MDH properties, by which to differentiate between the two forms, might profitably be undertaken in flounder during the course of gonad maturation.

Bigelow and Schroeder (1953) observed that winter flounder probably do not mature sexually until their third year (≥20 cm). We found this to be true for the female fish, but not always for the male (Table 3). Two R_4 males and, perhaps more significantly, eight spent males were considerably smaller and younger. Moreover, the overall biochemical picture corroborated the gonad-maturation rating obtained by gross examination, whatever the size of the fish. For example, values in partially-spent male gonads for G6PDH, PK, and LDH were in the same range as values for very immature gonads, but MDH values were still in the R_4 range, a combination not seen for any other stage. Additionally, levels of both carbonic anhydrase and acid phosphatase increased rapidly in the male gonad with the degree of gonad maturity (Table 4), and decreased even more rapidly in spent tissue. The pattern for these two enzymes clearly parallels spermatogenesis.

In summary, gametogenesis in this flounder is accompanied by low glycolytic and high pentose shunt activity, each of which in spent tissue returns to the levels of immature gonads. MDH and LDH in the male follow the course of glycolysis, and carbonic anydrase and acid phosphatase follow pentose shunt activity, which here reflects spermatogenesis. In the female, MDH and LDH patterns remain to be clarified; isoenzyme analysis during gonad maturation, especially of MDH, could be enlightening.

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