THE OSMOREGULATORY METABOLISM OF THE STARRY FLOUNDER, PLATICHTHYS STELLATUS

by

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B.A., DePauw University, 1950
M.S., University of New Hampshire, 1953

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CLEVELAND PENDLETON HICKMAN JR.
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ABSTRACT

Energy demands for osmotic regulation and the possible osmoregulatory role of the thyroid gland were investigated in the euryhaline starry flounder, *Platichthys stellatus*. Using a melting-point technique, it was established that flounder could regulate body fluid concentration independent of widely divergent environmental salinities. Small flounder experienced more rapid disturbances of body fluid concentration than large flounder after abrupt salinity alterations.

The standard metabolic rate of flounder adapted to fresh water was consistently and significantly less than that of marine flounder. In supernormal salinities standard metabolic rate was significantly greater than in normal sea water. These findings agree with the theory that energy demands for active electrolyte transport are greater in sea water than fresh water.

Thyroid activity was studied in flounder adapted to fresh water and salt water. Correlative with the higher metabolic rate of small flounder was the more rapid turnover and excretion of radioiodine and greater thyroid uptake of small than large flounder. Percentage uptake of radioiodine by the thyroid was shown to be an insensitive and inaccurate criterion for evaluating thyroid activity in different salinities because removal rates of radioiodine from the body and blood differed between fresh water and marine flounder.. Using thyroid clearance of radioiodine from the blood as a measure of activity, salt water flounder were shown to have much greater thyroid clearance rates and, hence, more active thyroid glands than flounder adapted to fresh water. The greater activity of the thyroid of marine flounder correlates with greater oxygen demands in sea water and suggests a direct or adjunctive osmoregulatory role of the thyroid gland of fish.

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I

INTRODUCTION

In recent years the problem of body fluid regulation in the lower vertebrates, in particular the fishes, has become of considerable interest. Perusal of a recent review (Black, 1957) shows that considerable research has been directed toward the elucidation of detailed mechanisms by which adult teleosts achieve osmotic independence from their environment. With the demonstration of basic regulatory mechanisms for both fresh and salt water fish, it became apparent that fundamental to their operation was the movement of charged particles from a lower to a higher potential - either primarily from the external environment to the internal milieu, in the case of fresh water teleosts, or primarily in the opposite direction in the case of marine teleosts. The certain involvement of active uphill particle movement means that a significant portion of cellular metabolism must be directed to the maintainance of this work. Thus, osmotic independence from the environment demands an expenditure of energy.

More recently, the role of the endocrines in salt and water metabolism of fishes has come under surveillance of an increasing number of workers. The subject is reviewed by Pickford and Atz (1957). No precise osmoregulatory function has been demonstrated conclusively for the endocrine glands, although the evidence is insufficient at present to preclude such a function for any of them. Often implicated in an osmoregulatory role is the enigmatic thyroid gland of teleosts. However, its precise action in this regard has remained obscure. Even more puzzling has been the inability of numerous workers to demonstrate in teleosts the fundamental calorigenic action of the thyroid of higher vertebrates.

The present investigation is a study of the osmoregulatory metabolism of a euryhaline teleost, the starry flounder, (Platichthys stellatus), subjected to varied environmental salinities, with particular reference to the role of the thyroid gland in osmotic regulation. The design of this investigation embodies

two assumptions, both hypothetical at the outset, that (a) using a euryhaline teleost, consistent quantitative differences in metabolic rate should be measurable in individuals adapted to different osmotic concentrations accordant with the osmotic gradient imposed and that (b) elucidation of an osmoregulatory role for the thyroid could be approached most successfully by looking for a general metabolic effect of the thyroid hormone rather than a specific action on one or more target organs. Thus, the hypothesis was proposed that if energy demands for osmotic regulation are mitigated or intensified by changing the ambient salinity, such changes should be reflected in a concomitant increase or decrease in both total metabolic rate and thyroid activity. In this thesis, experimental evidence is presented which demonstrate that energy demands of flounder are consistently and significantly greater in sea water than fresh water, and that the activity of thyroid gland as evaluated by thyroidal clearance rates of radioiodine from the blood is greater in sea water than fresh water adapted flounder.

The starry flounder was chosen as the experimental animal because in addition to possessing the prime attribute of euryhalinity, it is a hardy, rugged fish that lends itself exceptionally well to experimental treatment, it is relatively unexcitable, and could be readily collected from its marine environment during most of the year. Occasionally, other species of flatfish were collected, and isolated comparative experiments were performed when possible.

It is convenient to divide the presentation of this work into three sections: establishment of the osmotic capacity of the species in terms of its ability to regulate body fluid concentration in widely divergent salinities, the relative energy demands for regulation in these salinities and finally the part played by the thyroid gland.

II

EXPERIMENTAL ANIMALS

A. ENVIRONMENTAL RELATIONS OF STARRY FLOUNDER, LEMON SOLE AND SPECKLED SAND DAB

The flatfishes (Order Heterosomata) occupy a unique and interesting position among the Teleostei because of their remarkable departure from bilateral symmetry characteristic of the rest of the vertebrates. Except during the symmetrical larval stages prior to metamorphosis, flatfish are distinguished by having both eyes on the same side of the head. In the definitive adult body form, the eyed side of the body is pigmented and the blind side white. Flatfishes are typically demersal, both lying and swimming in a horizontal position.

Norman (1934) distinguishes five families of the Heterosomata, of which two are represented on the Pacific Coast of North America: Bothidae and Pleuronectidae. Only one genus of the Bothidae (left-eyed flounders) is found here, the genus Citharichthys. All of the rest of the flatfishes (14 genera in British Columbia waters) belong to the family Pleuronectidae, the right-eyed flounders.

This investigation is concerned primarily with the starry flounder, Platichthys stellatus, a euryhaline pleuronectid. In addition, an incomplete series of experiments were carried out on two partially euryhaline flatfishes, a pleuronectid, Parophrys vetulus, and a bothid, Citharichthys stigmaeus.

The starry flounder, Platichthys stellatus, is one of the most widely distributed of the Pacific Coast flatfishes, ranging from central California northward along the Canadian and Alaskan coasts and west along the Aleutian Islands to Tokyo Bay. It is also found in the Bering Sea and Arctic Ocean (Orcutt, 1950). It is a true coastal fish, inhabiting inlets, bays and estuaries of the North Pacific as well as the adjacent oceanic waters to 300 meters.

Juvenile flounders (1-6 cm.) are found abundantly intertidally in the spring and summer. Adults frequent somewhat deeper water, but are also common intertidally at certain seasons. Food is chiefly crustacea, polychaetes and molluscs. The spawning season of starry flounder is during the winter, from November through February in California (Orcutt, 1950), while perhaps a month later in British Columbia. Males reach maturity during their second year with a length of about 225 mm., females a year later with a length of about 250 mm.

The starry flounder's habit of entering brackish and fresh water all along the Pacific coast is well documented (e.g. Carl, 1937; Gunter, 1942; Hubbs, 1947; Clemens and Wilby, 1949; Orcutt, 1950; Roedel, 1953; Westrheim, 1955). Orcutt reports that a large number of small flounder 19 to 109 mm. in length (less than 30 grams) were collected in two California rivers. In British Columbia, several juvenile flounder, all less than 15 grams, were collected in a tributary of the Fraser River more than 20 miles above the river mouth. These specimens are deposited in the museum of the Institute of Fisheries, University of British Columbia. Presumably, large flounder also penetrate fresh water, for Westrheim (1955) reports that a number of tagged flounder, 6 to 27 inches in length (roughly 100 to 4000 grams) were recaptured inside the Columbia River, some as far as 20 miles upstream. In many cases of "fresh water" penetration, the fish may actually have been in brackish water; frequently a tongue of saline water underlies water of coastal rivers for some distance upstream. There is no question, however, that Platichthys stellatus voluntarily enters entirely fresh water on occasion.

The lemon sole, Parophrys vetulus, (known in American waters as the English sole) is limited in distribution to the Northeastern Pacific from the Gulf of Alaska to southern California (Norman, 1934). Juvenile forms (2-5 cm.) are commonly collected intertidally along with juvenile starry flounder. Older stages move into progressively deeper water. The adults most frequently are found on soft

sand or mud bottoms at depths of 60-100 meters (Ketchen, 1956). Food is mostly bottom-living invertebrates. Females reach maturity in their third or fourth year with a length of about 295 mm., the males in their second year at 250 mm. (Ketchen, 1947). As with the starry flounder, spawning occurs in the winter months of January through March. There is no record of lemon sole entering fresh water.

The speckled sand dab, Citharichthys stigmaeus, is an active flatfish ranging from southern California to southeastern Alaska (Clemens and Wilby, 1949). It is found in shallow water and to a depth of 80 meters. Because of its small size (reaching less than 17 cm. or 60 grams), it is of no commercial importance and has received virtually no attention from fishery biologists. Nothing is known of its early life history or food habits. The closely related, though larger Citharichthys sordidus (reaching 1000 grams) is a summer spawner (Arora, 1951) and observations made during the present investigation indicate that C. stigmaeus, also, spawns during the summer months. The speckled sand dab is a strictly marine fish, never entering fresh water, though it is not infrequently found in the intertidal zone. It was often taken during the spring and summer on Vancouver beaches by the author when the spring run-off from the nearby Fraser River had lowered the salinity to near isotonicity with the fishes' body fluids.

Platichthys stellatus is not the only euryhaline flatfish. The European flounder Pleuronectes flesus is well known for its habit of entering fresh water. Both this species and Pleuronectes platessa (plaice) penetrate extensively into the low-salinity Baltic Sea, but the latter is unable to live in fresh water. Henschel (1936) established that plaice could not indefinitely withstand a salinity below $8^{o}/oo$ (Δ .43). Norman (1934) lists two additional pleuronectids that enter fresh water: Liopsetta glacialis, the Arctic flounder which is distributed along the Arctic shores of Russia, Alaska and Canada, and Rhombosolea retiaria, the black flounder of New Zealand.

Euryhaline bothids are known: Citharichthys stamflu of West Africa and Citharichthys gilberti of the Pacific coast of tropical America from Lower California to Peru (Norman, 1934). Both of these species have been recorded in fresh water.

B. COLLECTION AND CARE OF FISH

Experimental work was carried out over a two year period from summer, 1956 to spring, 1958. During most of the work, the laboratory facilities of the Vancouver Public Aquarium were utilized. A constant supply of both dechlorinated fresh water and filtered sea water was available. The salinity of the sea water in the Aquarium varied seasonally. During the winter the salinity usually remained high - 25-27°/oo, - but in the early summer it dropped to as low as 16°/oo because of the heavy discharge of the nearby Fraser River at that time of year. The salinity rarely rose above 20°/oo until fall.

During 1956, flounder were collected at frequent intervals by means of a beach seine at Locarno Beach, Vancouver. The following year, the service of a small otter trawl was successfully employed, and thereafter, all flounder were caught by this method on the North Bank of the Fraser River, Steveston, British Columbia. Both of these areas are characterized by low salinities in the summer-time - the Locarno Beach area as low as 10°/oo and the North Bank of the Fraser as low as 6°/oo. The latter is largely protected from the full influence of the river by a jetty which extends several miles offshore from the river mouth.

Flounder were transported to the laboratory in 10 gallon containers with aeration. Fish were kept in tanks containing about 130 liters of running sea water at a temperature within 1°C. of the environmental temperature in which the fish were captured. After 2 or 3 days in running water, the flounder were transferred to tanks containing 130 liters of aerated water adjusted to the

desired experimental salinity. Temperature was maintained within ±1.0°C. of the environmental temperature by means of running fresh water cooling tubes. Water was changed at intervals when any sign of fouling appeared, but this was rarely any problem if the fish were given the initial two-day period in sea water for the elimination of metabolic wastes. The tanks were partially covered, but daylight was not excluded. The fish appeared to thrive under these conditions and virtually no mortality occurred between the time of capture and the end of the experiment (two weeks or less).

Sand dab and lemon sole were captured at Locarno Beach, occasionally with flounder in a beach seine, but these species usually remain in somewhat deeper water and were not readily taken by this method. To capture these fish in deeper water in the same area, a small beam trawl was used. However, the capture of fish of sufficient numbers and of a suitable size range was never easy and the supply proved to be unpredictable. Sand dab and lemon sole were transported to the laboratory and treated in similar fashion to the flounder.

Since the metabolism of foodstuffs forms a large portion of the total calorific output of animals, it is usually found advantageous to fast experimental animals before measuring standard[1] metabolism. Accordingly, none of the flatfish were fed in the laboratory. There is no evidence that the drop in metabolic rate, caused by the cessation of food assimilation and protein storage for growth, would in any way interfere with the fish's capacity to perform osmotic work. In the present experiments, although starved flounder lived for 1½ months in the summer

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1. Basal metabolism, a term in common usage by medical physiologists and clinicians, refers to the metabolic rate of a resting (but not sleeping) and fasted animal, removed from discomfort and distracting influences. Because the metabolism of vertebrates can be lowered from the "basal" rate by drugs or sleep, Krogh (1914) suggested the term standard metabolism for the energy requirements under normal resting conditions. Standard metabolism would seem to be a more accurate term and has been adopted by most comparative physiologists studying energy metabolism in animals.

and about 3 months in the winter before appreciable mortality began, all experimental work was begun at least within 10 days (usually within 4 days) and terminated within 15 days after collection of the fish.

Thermal acclimation is one of the most important factors in oxygen consumption comparisons of fish measured at different times. If the ambient environmental acclimation temperature is changed, there will occur a measurable systematic change in the metabolic rate to a new level. In an effort to eliminate any shift due to changes in thermal acclimation, the fish were held in temperature controlled tanks within $\pm 1.0^{\circ}C.$ of the surface temperature measured at the collection locale. However, surface temperatures are not always representative of bottom temperatures (though nearly so in the marine intertidal zone) and may fluctuate considerably with tidal movements. In addition, there is the possibility that the flatfish which are known to carry on diurnal migrations, may actually be acclimated to a colder deep water temperature. Thus the thermal history of the flatfish was, within certain limits, unknown. Changes in temperature acclimation, with concomitant shifts in the fishes' metabolic response to the experimental temperature was then always a possible variable. At the very extreme, however, the discrepancy between environmental and laboratory temperature did not exceed $1.5^{\circ}C.$, hence the resulting metabolic shifts due to any departure between these temperatures must be small.

III

THE OSMOTIC RESPONSE OF STARRY FLOUNDER TO CHANGES IN ENVIRONMENTAL SALINITY

In fishes, the principle organs for osmotic exchange with the environment are the kidneys and the gills. Fresh water fish depend primarily for the prevention of overhydration on the excretion of a copious and dilute urine, whereas the kidney is more of a liability than an asset to the marine teleost. In the latter there have developed in the gill adaptive cells capable of concentrating and excreting ions to the exterior. In spite of the fundamental opposition of direction of active salt and water exchange in fresh and salt water fishes, (overhydration vs. dehydration) both regulate the osmolar concentration of their body fluids at comparable levels: fresh water fishes at Δ0.5 to 0.7°C. and marine fishes at about Δ 0.7 to 0.9°C. (Black, 1957). Reported values for body fluid osmolarity of fishes vary greatly between species and often within a species. Interspecific variations are due to several contributing factors such as error in various methods of measurement and abnormal changes in plasma osmolarity due to "laboratory diuresis" (Forster, 1953). In addition, body fluid osmolarity is never maintained absolutely constant even under relatively stable environmental conditions, and a certain amount of lability is to be expected, particularly if the osmotic gradient changes.

The capacity of fishes to adapt to vicissitudes in the tonicity of the environment varies markedly. It is generally recognized that the terms stenohaline, referring to animals that can tolerate only a narrow environmental concentration range, and euryhaline, referring to animals tolerating a wide salinity range, are inadequate for categorizing all fishes. Most oceanic marine fishes are more or less stenohaline, but toward coastal areas there occur increasing numbers of fishes that tolerate dilute sea water. In the intertidal region, in estuaries and at river mouths marine fishes are found living

comfortably in near-fresh or even fresh water. Thus, the above terminology will not cover the relatively frequent case of the marine fish that enters brackish water but cannot survive in fresh water. The degree of euryhalinity of a species may depend to some extent on the degree of dilution or concentration of total body electrolyte that can be tolerated, but ultimately it is contingent upon the capacity of the organs of exchange to adapt to changes or reversal of the concentration gradient.

Evaluation of euryhalinity usually is based on the ability of fish to survive a wide range of salinities. Such a criterion is crude, for it cannot reveal the extent of disturbance of body fluid composition and concentration, hence the degree of perfection of homeostatic mechanisms. Presented in this section is a precise method for describing the ability of an aquatic animal to regulate under a wide range of concentration gradients by a technique hitherto unused by fish physiologists.

A. DETERMINATION OF BODY FLUID CONCENTRATION

1. Melting-point Determination.

Total osmotic pressure of serum and urine was determined by a melting-point method similar to that used by Gross (1954) for the measurement of osmotic pressure changes in the body fluids of a sipunculid. This method involves comparison of the time of melting of frozen solutions of unknown melting point with solutions of known melting point, when allowed to warm very slowly and linearly in a cold brine bath. Because only enough solution (.01 - .001 ml.) is needed to fill a small portion of a capillary tube, the method is particularly desirable where small quantities of fluid are available. For this study, this method offered the following advantages: (a) it is applicable for osmocentration measurements of body fluids of small fish (less than 10 grams) as well as large, (b) urinary catheterization is unnecessary for urine collection since only a drop of

urine is needed, an amount easily expressed by gentle pressure over the urinary bladder, and (c) many of the problems encountered in freezing-point determinations are eliminated, e.g. coagulation and supercooling.

The brine bath consisted of a 2-liter capacity plastic box insulated with cork and rock wool. Windows were provided on the top and bottom of the chamber for illumination and observation of the tubes. Standard sodium chloride solutions with melting points of about -2°. -1°, -0.5° and 0°C. were prepared and the exact osmotic concentrations of each determined to an accuracy of $.005^{\circ}$C. by the freezing-point method. In an experimental run, capillary tubes containing approximately equal quantities of the unknowns were quick-frozen on dry ice and placed side by side on a rack in the brine bath at an initial temperature of -9.0°C. Beside these were placed capillary tubes containing equal quantities of the four standard solutions, similarly frozen on dry ice. The brine bath was then covered and stirred gently during the period of warming. Warming was rapid initially, but soon slowed. During the critical period (-2.0 to 0°C.) the temperature rose about 1°C. every 35 minutes in a nearly linear fashion. The tubes were watched with a long-arm binocular microscope and the time of melting of both standard and unknowns recorded by marking a kymograph drum with signal magnets arranged for the purpose. Then, by plotting and fitting a line through the points, the melting-points of the unknowns could be interpreted relative to those of the standards. The method used here had an accuracy to within $.01^{\circ}$C., a little less than the accuracy possible to achieve by careful freezing point determinations ($.005^{\circ}$C.).

2. Experimental Procedure.

It has been shown frequently that marine fish held in overcrowded conditions in the laboratory or subjected to handling develop a "laboratory" or "osmotic diuresis" that may persist for a long period of time (Grafflin, 1931; Pitts, 1934; Clarke, 1934; Meyer, 1948; Forster, 1953; Forster and Berglund,

1956). Evidence indicates that a spontaneous increase in urine flow occurs immediately after capture whether or not subsequent handling or treatment is imposed. However, cannulation of the urinary bladder for renal clearance studies seems to augment diuresis considerably. Most apparent physiological changes are the rise in osmoconcentration of both blood and urine and finally isotonicity between blood and urine. The selective electrolyte reabsorption by the kidney tubules breaks down so that in addition to other changes, an excessive renal loss of chloride occurs. The reason for the onset of laboratory diuresis is not known other than that it is the result of the trauma imposed during capturing, holding and handling. In this investigation, single urine samples were collected from each fish, thus eliminating two important contributing factors to severe laboratory diuresis: bladder catheterization and the continual handling necessary for serial sampling of individual fish.

After collection, fish were held for three days in flowing sea water at a temperature of 14–15°C. At the beginning of the experiment, a group of flounder were transferred rapidly from the holding tank to the desired salinity. Controls (flounder maintained in normal, 25°/oo sea water) also were transferred to a similar aquarium so that effects due to handling in transfer would be the same in both control and experimental fish. At selected intervals of time following transfer, fish were removed, rinsed in fresh water and blotted dry. A urine sample was collected by touching the tip of a capillary tube to the urinary papillae and pressing gently over the urinary bladder. The drop of urine was tapped to the center of the tube, the tube sealed at both ends with a heavy inert grease (Nevastane, Heavy X, Keystone Co.) and immediately placed on dry ice. Blood was collected by puncturing the dorsal aorta above and slightly posterior to the center of the coelom with a narrow, sharp-pointed scalpel blade. Because of the lateral compression of flatfish, the dorsal aorta lies relatively near the surface and may be easily punctured from the side of the animal without entering either the coelom or spinal cord. By

inverting the animal, blood was allowed to flow onto a clean glass slide and left undisturbed until it had clotted (20-50 seconds). Serum was drawn into the capillary tube and the tube sealed and frozen. Melting-points were determined either immediately or the tubes were stored briefly in a brine solution at -10°C. until such time as the measurement could be made.

B. RESULTS

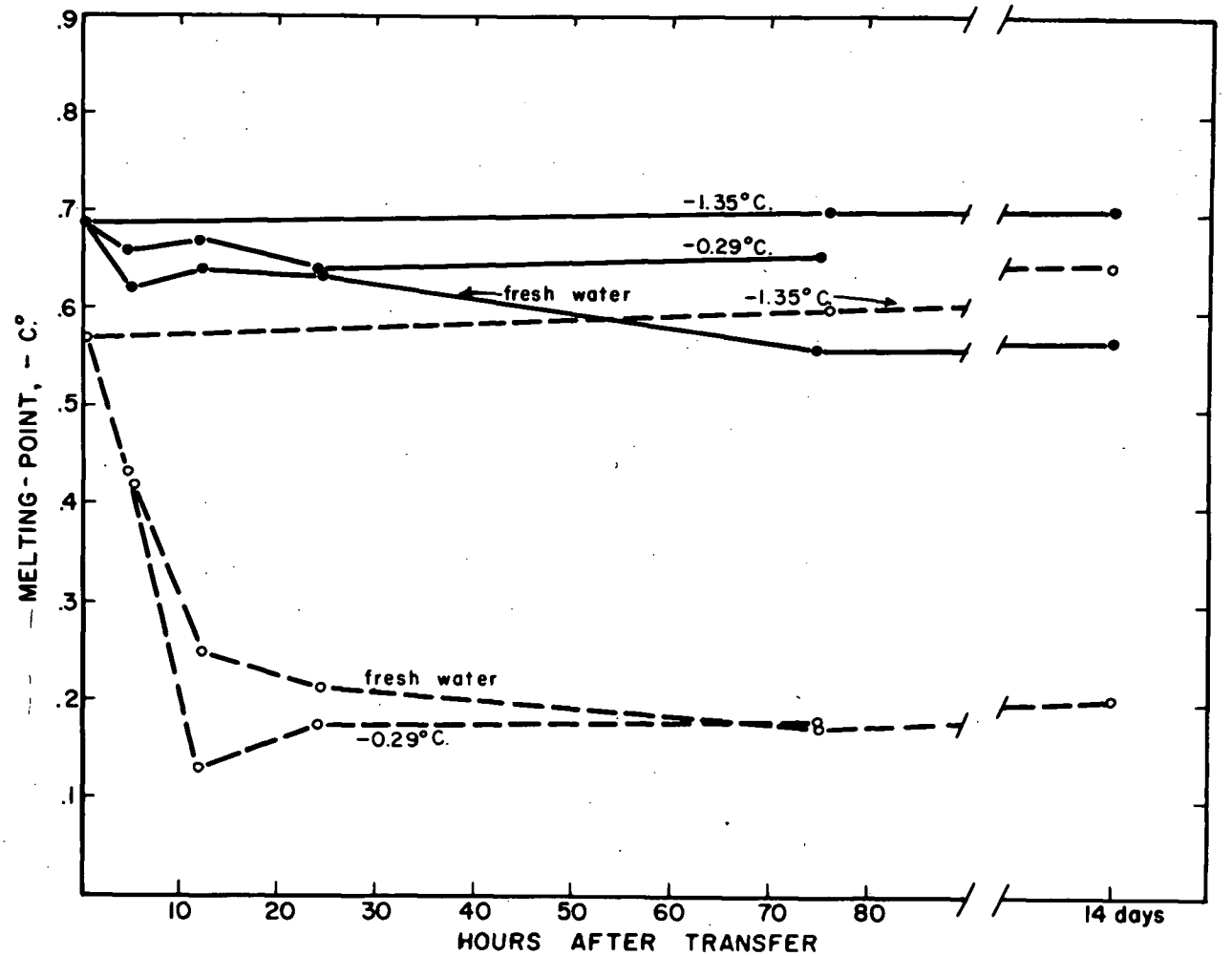
1. Starry Flounder in Normal Sea Water.

At the outset of the experiment the osmoconcentration of the serum of flounder in 25°/oo sea water averages Δ 0.69°C. (Fig. 1a, Table I). At 76 hours the average serum osmolarity has increased to Δ 0.702°C. and to Δ 0.706°C. at 14 days. The urine melting-point was invariably lower than the serum melting-point of the same fish, as has been repeatedly demonstrated for other marine teleosts (Dekhuyzen, 1905; Smith, 1932; Martret, 1939; Forster, 1953; Brull and Cuypers, 1954; Forster and Berglund, 1956).

It is notable that with the large increase in variability of serum and urine concentration at 14 days, urine always remains hypotonic to the serum when values are compared on an individual basis. In comparing relative degrees of hypotonicity of urine to serum, the urine: serum melting-point ratio is useful; these values have been calculated and are included in Table I. The average U/S ratios of the initial and 76 hour samples are essentially the same (.834 and .835), but the average 14 day U/S ratio has increased to .91. Forster (1953) and Forster and Berglund (1956) have shown that the onset of laboratory diuresis is accompanied by a shift in total electrolyte composition of plasma and urine and an increase in urine flow and loss of chloride, all of which contribute to a marked increase in tonicity of both urine and extracellular fluid. They noted a progressive rise in the urine:plasma ratio during laboratory

Figure 1a. Average melting points of serum (solid lines) and urine (broken lines) of Platichthys stellatus. transferred abruptly from sea water of 25 °/oo (Δ 1.35°C.) to dilute sea water of 5.45 °/oo (Δ 0.29°C.) and fresh water. Individual values summarized in Table I.

Figure 1b. Average melting points of surum (solid lines) and urine (broken lines) of Platichthys stellatus transferred abruptly from sea water of 25 °/oo (Δ 1.35°C.) to concentrated sea water of 46 °/oo (Δ 2.49°C.). Individual values summarized in Table I.


.9
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— MELTING-POINT, - C°
-1.35°C.
-0.29°C.
fresh water
-1.35°C.
fresh water
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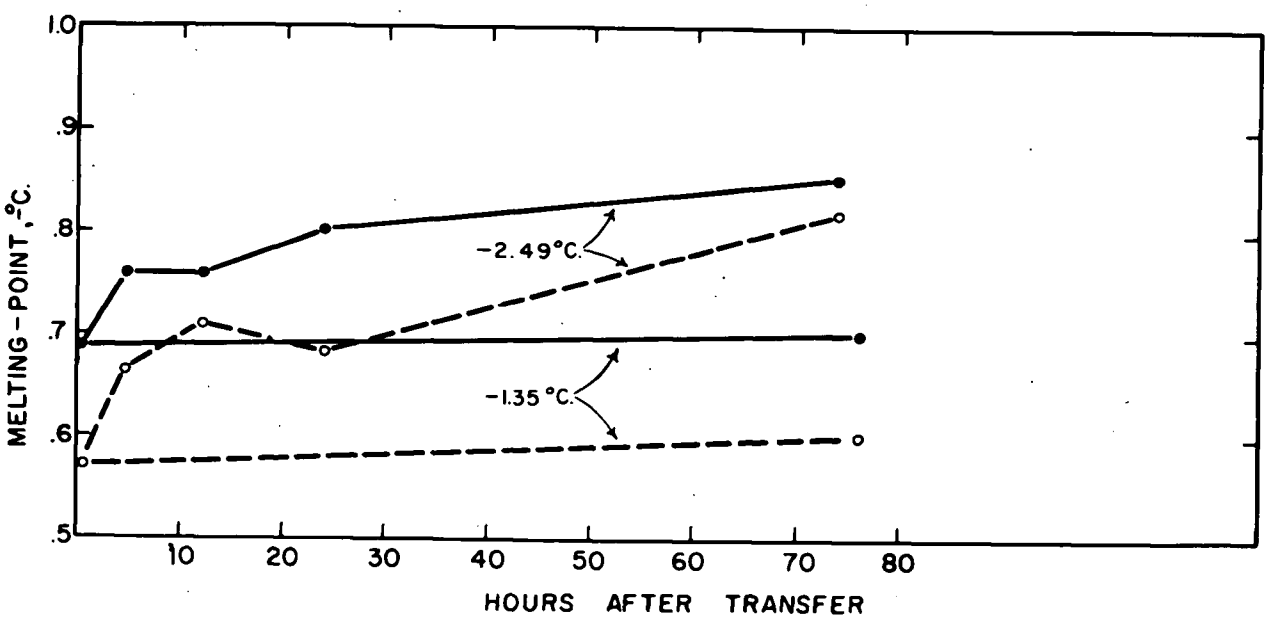
20
30
40
50
60
70
80
14 days
HOURS AFTER TRANSFER


1.0
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MELTING-POINT, °C.
-2.49°C.
-1.35°C.
10
20
30
40
50
60
70
80
HOURS AFTER TRANSFER

Table I. Melting points of serum and urine and urine:serum ratios of Platichthys stellatus in the control salinity of 25‰, and after transfer to 46‰ sea water, 5.45‰ sea water and fresh water.

Salinity 25‰

Time hrs.	Weight gm.	Melting Point Serum -°C.	Urine -°C.	U/S
0	13.8	.68	.58	.852
	17.3	.705		
	22.5	.687		
	28.6	.705	.58	.823
	35.1	.672	.555	.826
	Average	.689	.571	.834
76	10.1	.71	.62	.873
	10.4	.70	.59	.833
	18.2	.71	.60	.845
	48.2	.72	.58	.805
	57.5	.76	.622	.818
	Average	.702	.602	.835
14 days	7.2	.645	.602	.933
	12.3	.748	.715	.955
	.515	.70		
	21.0	.763	.672	.854
	45.6	.675	.608	.90
	Average	.706	.644	.91

Salinity 46‰

Time hrs.	Weight gm.	Melting Point Serum -°C.	Urine -°C.	U/S
4½	8.5	.778	.66	.848
	12.2	.89	.68	.775
	15.9	.73	.66	.905
	39.5	.715		
	139.2	.68		
	Average	.759	.666	.843
12	9.1	.78	.745	.955
	12.7	.80	.697	.871
	25.9	.73	.696	.953
	33.5	.752	.707	.94
	130.7	.73		
	Average	.758	.711	.930
24	8.7	.792		
	10.8	.825		
	13.1	.845		
	31.0	.792	.685	.865
	210.1	.765	.685	.895
	Average	.803	.685	.88
74	10.5	.912	.89	.976
	12.5	.845	.835	.99
	25.4	.835	.765	.915
	34.7	.87	.837	.962
	77.3	.81	.765	.945
	Average	.854	.818	.958

Table I (Continued)

Salinity 5.45°/oo

Time hrs.	Weight gm.	Melting Point Serum -°C.	Urine -°C.	U/S
4¾	6.9	.630		
	13.0	.662		
	15.3	.68	.60	.883
	48.1		.125	
	93.2	.668	.575	.86
	Average	.66	.433	.871
12	6.7	.695	.147	.212
	17.7	.646		
	23.1	.672	.155	.231
	57.9	.662	.115	.174
	58.0	.68	.105	.154
	Average	.671	.131	.193
24½	9.2	.645	.30	.455
	11.5	.642	.20	.311
	18.7	.65	.19	.292
	67.7	.662	.08	.121
	170.0	.612	.11	.18
	Average	.642	.176	.271
75	11.8	.688	.311	.451
	14.7	.59		
	16.3	.634	.616	.971
	25.4	.679	.14	.206
	77.3	.69	.088	.127

Fresh Water

Time hrs.	Weight gm.	Melting Point Serum -°C.	Urine -°C.	U/S
5	7.4	.58	.515	.888
	18.5	.642	.085	.132
	19.4	.612	.435	.71
	45.3	.642	.545	.849
	68.5	.63	.515	.817
	Average	.621	.419	.679
12	10.0	.635	.264	.417
	15.6	.644	.288	.447
	19.5	.61		
	21.7	.65	.304	.458
	76.5	.66	.134	.203
	Average	.64	.248	.381
24	9.3	.66	.254	.384
	9.7	.622	.192	.308
	20.2	.665	.335	.504
	29.4	.615	.142	.23
	74.3	.615	.15	.244
	Average	.635	.214	.334
75	12.7	.505	.295	.584
	14.1	.597	.265	.444
	19.9	.535	.107	.20
	35.5	.575	.107	.186
	64.6	.587	.107	.199
	Average	.559	.176	.322
14	3.6	.544	.47	.865
	11.0	.583	.105	.18
	17.3		.105	
	50.0	.58	.145	.25
	Average	.569	.206	.431

diuresis and eventually blood and urine become isotonic. Inasmuch as the U/S ratio of flounder in this study increased not at all between 0 and 76 hours and only slightly at 14 days, it is unlikely a laboratory diuresis developed during the experiment. It is conceivable, nevertheless, that a change had occurred in the U/S ratio in the 3-day interval between capture and initial sampling. Forster, (1953), working with three marine species, two aglomerular and the other glomerular, found urine:plasma freezing point ratios of samples collected immediately after capture to vary between 0.81 and 0.87. These values compare closely with urine:serum ratios obtained for the flounder. If we assume Forster's findings are representative of the normal condition, the U/S ratios obtained for flounder in the present investigation fall in the normal range for marine teleosts.

2. Starry Flounder in Hypotonic Media:
Regulation Against Overhydration and Salt Depletion.

a. Concentration disturbances

Abrupt transfer to hypotonic media results in an immediate dilution of the blood of flounder (Fig. 1a). Since water freely traverses cellular membranes the net result is a dilution of both cellular and extracellular fluids. The average serum osmolarities of flounder in both fresh water and hypotonic sea water are lower at 5 than at 12 hours, suggesting that the initial influx of water into the extracellular space overwhelms the capacity of the kidney to excrete the excess fluid. A brief recovery occurred at 12 hours and was followed by a slower drop in the body fluid concentration until a balance was struck between water influx and output. This occurred within 24 hours for flounder in 5.45o/oo sea water, but the concentration continued to drop in fresh water flounder until equilibrium was reached, sometime between the first and third days following transfer.

The concentration of the urine decreased markedly during the first day (Fig. 1a). Surprisingly, the drop in urine concentration of flounder in

dilute sea water is initially greater than that of fresh water flounder, a phenomenon that must be presented without an attempt at explanation. As would be expected, urine concentration reached equilibrium at about the same time as did the blood concentration, i.e., 24 hours for flounder in dilute sea water and about 3 days for fresh water flounder. The slight increase in osmolarity of the serum and urine of the flounder in fresh water between the third and fourteenth days is probably the result of a net retention of solutes with inanition as demonstrated with goldfish by Meyer, Westfall and Platner (1956) and Jorgensen and Rosenkilde (1956).

b. Volume disturbances

Starry flounder tolerate abrupt transfer from sea to fresh water with absolutely no outward appearance of distress or asthenia. In fresh water they remain alert and vigorous under the added imposition of starvation for many weeks. Some changes in body volume and, as has already been discussed, concentration, do occur which have significance in assessing the actions of the osmotic stress imposed.

Measurements of weight changes were not carried out in this study. Henschel (1936) has, however, followed weight changes of the euryhaline Pleuronectes flesus (very similar to Platichthys stellatus) after transfer from a hypertonic to hypotonic medium. No change in weight occurred when transferred to 8°/oo (Δ 0.42°C.) from 16°/oo (Δ 0.95°C.) but some increase in weight occurred at 4°/oo (Δ 0.21°C.), due to water loading. Henschel also made an interesting comparison between the flounder and less adaptable plaice, P. platessa. In 8°/oo sea water the plaice gained weight over the 16°/oo controls and in 4°/oo they gained as much as 20% in body weight before death within 3 days. In other experiments he found that plaice lost salt as rapidly as they gained water, resulting in a rapid fall in serum osmolarity and death with little weight change. Whereas flounder were able to excrete the excess

water by increasing urine flow, urine production of plaice in the 4°/oo sea water actually decreased or even stopped, thus vastly increasing the disparity between water influx and excretion. In addition, Henschel found that flounder transferred to hypotonic solutions stopped drinking water, while plaice continued. Thus P. flesus is many times better suited to life in low salinities than P. platessa.

Starry flounder in the present study were observed to increase body volume somewhat after transfer to fresh water, but no excessive hydration was apparent until after several weeks of starvation at 15°C. Inanition became severe after 5 to 6 weeks in fresh water, when mortality began. The immediate and direct cause of death appeared almost invariably to be the result of a breakdown in osmotic regulation, caused by acute caloric starvation. A noticeable edema occurred and was accompanied by a stiffening of the body and a marked decrease in swimming activity when disturbed. Moribund fish were occasionally so stiff that they could hardly be forcefully bent when picked up.

Also characteristic of osmotic failure were cardiovascular disturbances. Collection of blood samples became increasingly difficult, suggesting a marked decrease in blood volume and/or cardiac output, producing a near circulatory stasis. Such critical changes in circulation occurred during the terminal stages of osmotic failure, but some small decrease in the effective blood flow was occasionally witnessed in healthy fresh-water flounder and appeared to be a normal consequence of the relative over-hydration of these animals.

3. Starry Flounder in Supernormal Hypertonic Media: Regulation Against Dehydration and Salt Excess.

The blood and urine osmolarities of flounder abruptly transferred to concentrated sea water of 46°/oo (Δ 2.49°C.) from the control salinity of 25°/oo (Δ 1.35°C.) quickly rise (Fig. 1b, Table I). The average serum melting-point drops from Δ 0.69 to Δ 0.76°C. during the first 4½ hours, a decrease of about 10% (10% increase in total osmotic pressure). Between 4½ and 12 hours the concentration changes very little, but is then followed by a gradual continuous rise to 74 hours, when the experiment was terminated. Whether the brief pause between 4½ and 12 hours has special significance is not known, though a similar "recovery" period was noted in the progressive decrease in blood concentration of flounder transferred to fresh water.

4. The Effect of Body Size on Alterations in Body Fluid Osmolarity After Abrupt Changes in Environmental Salinity.

To assess the effect of size on the rapidity of concentration changes affected by abrupt salinity alterations, the individual melting points of serum were plotted on a linear axis against body weight on a logarithmic axis. An example is shown in Figure 2. The progressive increase in serum melting-points of flounder abruptly transferred to concentrated sea water are plotted for each sampling period following the transfer. Included are the urine melting-points (open circles). From each eye-fitted line through the values, 2 points were graphically derived representing the serum melting points of a 10 gram and a 60 gram starry flounder and plotted against time after transfer (Fig. 3). It is evident from Figure 3 that the osmotic disturbance following a salinity change is considerably greater in smaller flounder. The body fluid concentration of smaller flounder is shifted more rapidly and to greater degree than large flounder, in the direction of the osmotic concentration of the external media, i.e., serum osmolarities are greater in concentrated sea water and less in fresh water in smaller individuals.

Figure 2. Melting points of serum (•) and urine (o) of Platichthys stellatus transferred abruptly to concentrated sea water of 46 °/oo (Δ 2.49°C.).

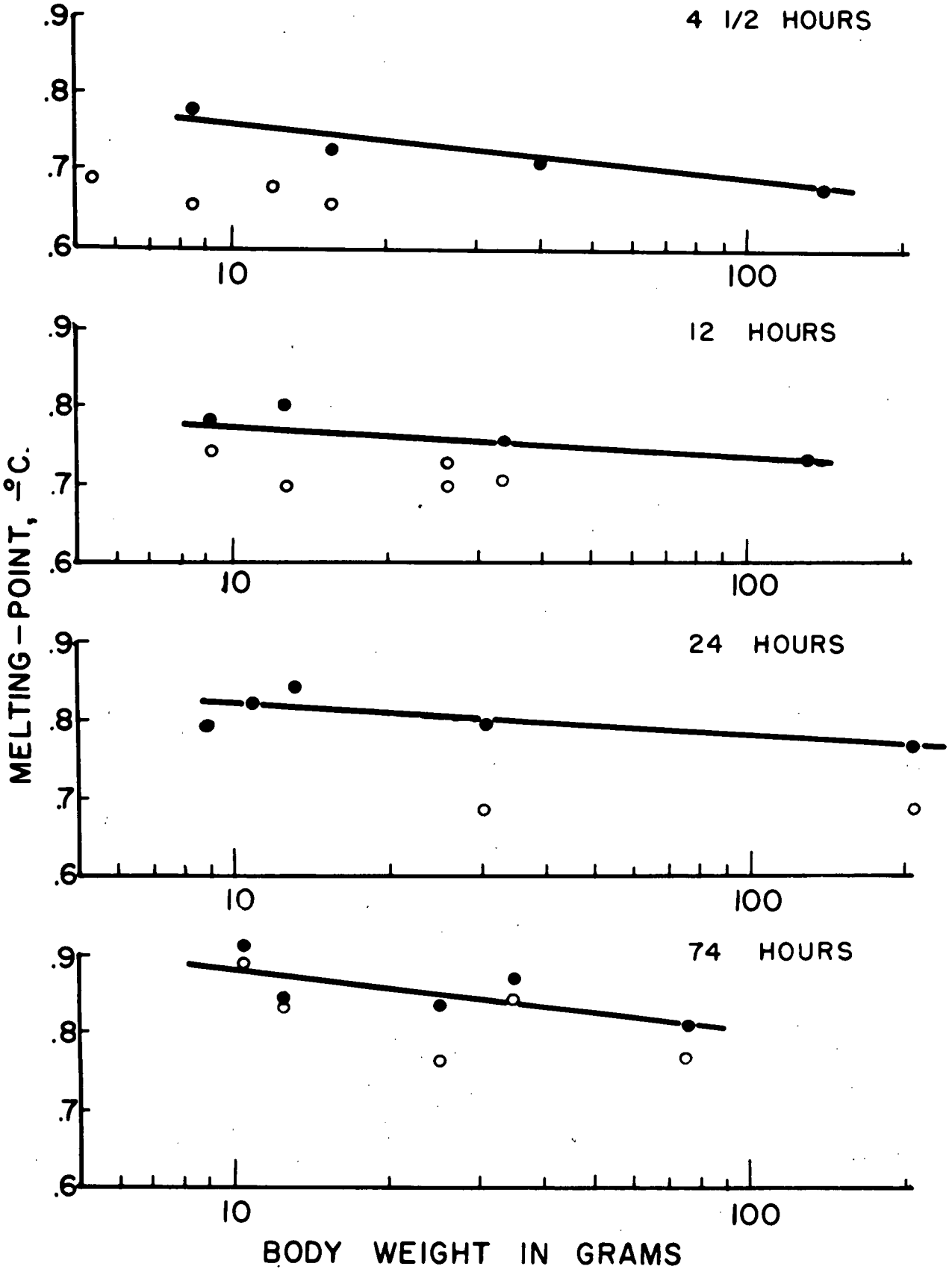
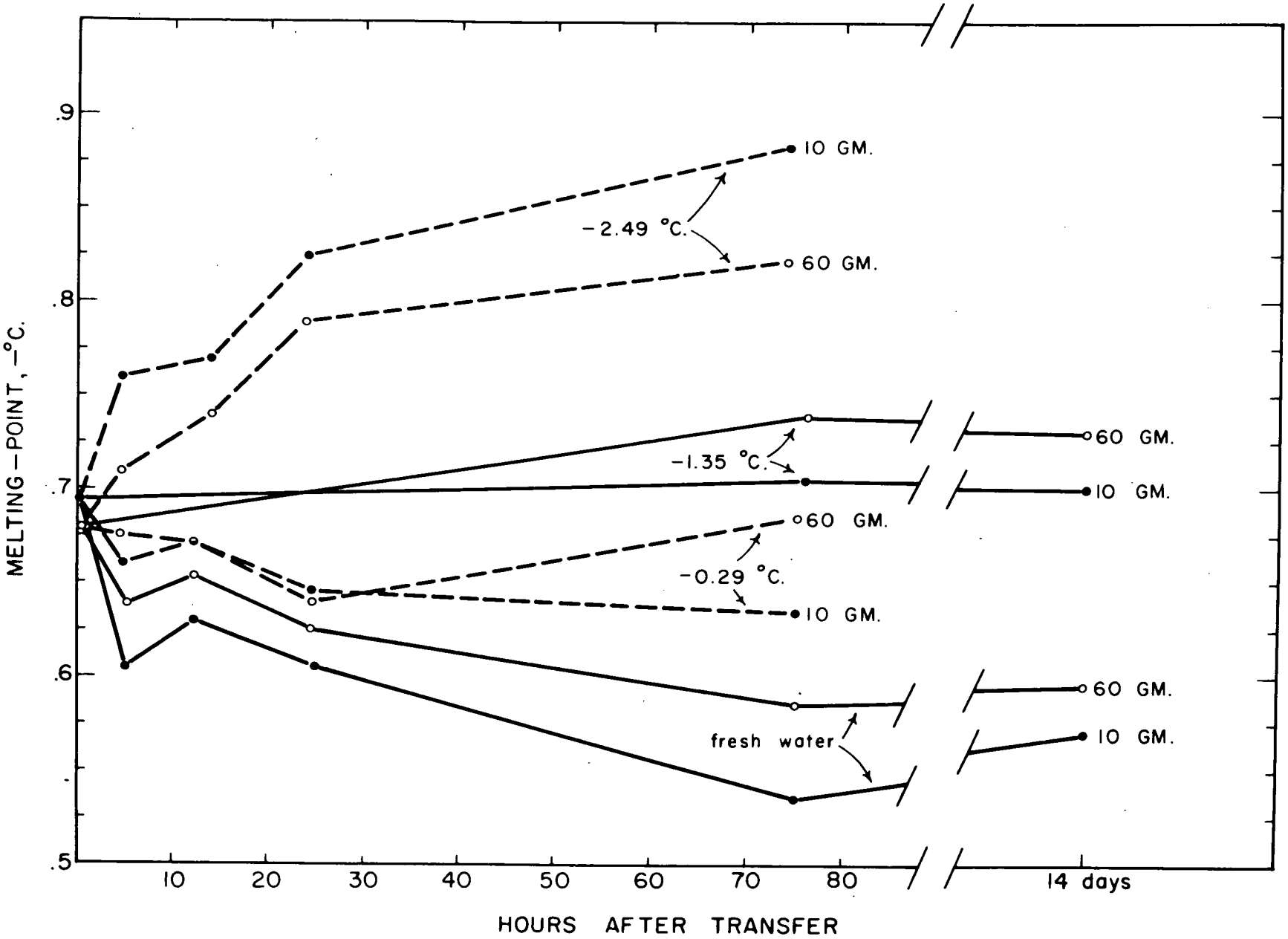

4 1/2 HOURS
12 HOURS
24 HOURS
74 HOURS
.9
.8
.7
.6
10
100
MELTING-POINT, °C.
BODY WEIGHT IN GRAMS

Figure 3. The effect of body size of the rapidity of change of serum melting point of Platichthys stellatus transferred abruptly from sea water of 25 °/oo to concentrated sea water of 46 °/oo, to dilute sea water of 5.45 °/oo and to fresh water. The points representing 10 gram (•) and 60 gram (○) starry flounder were graphically derived from eye-fitted lines through individual serum melting points for each sampling period of each experimental salinity (see Figure 2 for example). Individual values are summarized in Table I.


.9
.8
.7
.6
.5
MELTING-POINT, -°C.
10 GM.
-2.49 °C.
60 GM.
60 GM.
-1.35 °C.
10 GM.
60 GM.
-0.29 °C.
10 GM.
60 GM.
fresh water
10 GM.
10
20
30
40
50
60
70
80
14 days
HOURS AFTER TRANSFER

C. COMMENT

It has been suggested (Gordon, 1957) that euryhaline teleosts may be divided into two groups with respect to their ability to regulate the osmoconcentration of the body fluids at the same or at different levels when transferred from hypo- to hypertonic media or the reverse. Certain teleosts apparently experience only transitory changes in body fluid osmolarity when subjected to salinity changes. The stickleback Gasterosteus (Gueylard, 1924; Koch and Heuts, 1943) and the killifish Fundulus heteroclitus (Burden, 1956) maintain essentially the same plasma osmolarities or chloride concentration in either hypo- or hypertonic media.

For nearly all of the Salmonoids examined however, rather large shifts (12 to 25%) occur in plasma concentration levels of fish acclimated to hypo- and hypertonic media, the shift tending to decrease the concentration gradient between the internal and external milieu (Greene, 1904, 1926; Benditt et. al. 1941; Fontaine, 1943, 1948; Fontaine and Koch, 1950; Fontaine, Callamand and Vibert, 1950; Kubo, 1953; Gordon, 1957). However, in this laboratory, Houston (1958) has demonstrated that the plasma chloride concentration of steelhead trout transferred abruptly to sea water from fresh water may return to pre-transfer levels. Several authors have found appreciable lability in serum and tissue concentration of the European eel, Anguilla vulgaris (Portier and Duval, 1922; Duval, 1925; Boucher-Firly, 1935) and the American eel, A. rostrata (Smith, 1932) exposed to fresh and salt water.

The ability of some fish to control body fluid osmolarity at one level regardless of external variations does not necessarily imply that these fish osmoregulate more efficiently than those that allow substantial internal osmotic fluctuations in the direction of the external shift. On the contrary, whereas the former possess very effective and capable homeostatic mechanisms, such rigid homeostasis is considerably less efficient in terms of thermodynamic work than the

less advanced method of reducing the osmotic gradient. As Potts (1954) has pointed out, the most important mechanism for reducing the osmotic strain imposed on a marine animal entering brackish water is to decrease the concentration of the blood. It should be emphasized that all euryhaline teleosts are still homoiosmotic regulators and that the osmotic lability illustrated by some is only relative with fluctuations always held within certain strict limits. In this investigation blood melting-points dropped from $-0.70^{\circ}C.$ in salt water flounder to $-0.55^{\circ}C.$ in fresh water flounder adapted 75 hours - a 20% decrease. Henschel (1936) transferred the euryhaline European flounder, Pleuronectes flesus from dilute sea water of $16^{o}/oo$ (Δ $0.95^{\circ}C.$) to a hypotonic salinity of $5\text{-}6^{o}/oo$ (about Δ $0.3^{\circ}C.$), and measured a 10% decrease in the freezing point depression of the blood, Δ 0.58 to $0.52^{\circ}C.$ Under the same conditions the plaice, Pleuronectes platessa, a hypo-osmotic regulator, rapidly lost salt and soon died at a body fluid tonicity approaching that of the external milieu.

The division of body fluid compartments and the chemical and physical forces that determine movements and distribution of fluids and solutes are essentially the same throughout the vertebrates. Hence, reference to the vast mammalian literature is valuable to an interpretation of fluid balance in fishes. Excellent comprehensive treatments on body fluid dynamics are found in the writings of Peters (1953), Elkinton and Danowski (1955), and Gamble (1958).

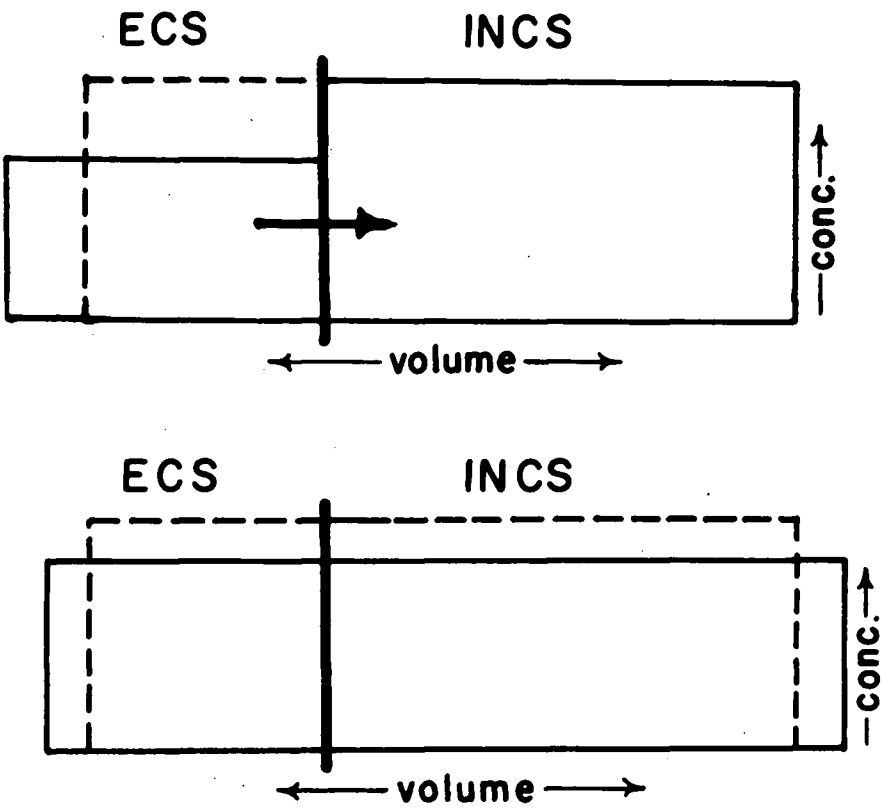
The disturbances of the body fluids seen in the flounder transferred to fresh water can be illustrated with Darrow-Yannet diagrams (Fig. 4) adapted from Elkinton and Danowski (1955). Osmotic water gained by the flounder enters via the gills and oral membranes into the plasma and interstitial fluid, together known as the extracellular space. It is this compartment, "le milieu interieur" of Claude Bernard, that acts as a buffer between the vicissitudes of the external environment and the cells. Normally the osmotic pressures between the interstitial fluid and intracellular fluid are equal though the relative composition

Figure 4. A diagrammatic representation of fluid shifts attending concentration and volume disturbances of flounder transferred to fresh water. The extracellular and intracellular spaces are separated by the heavy vertical line.

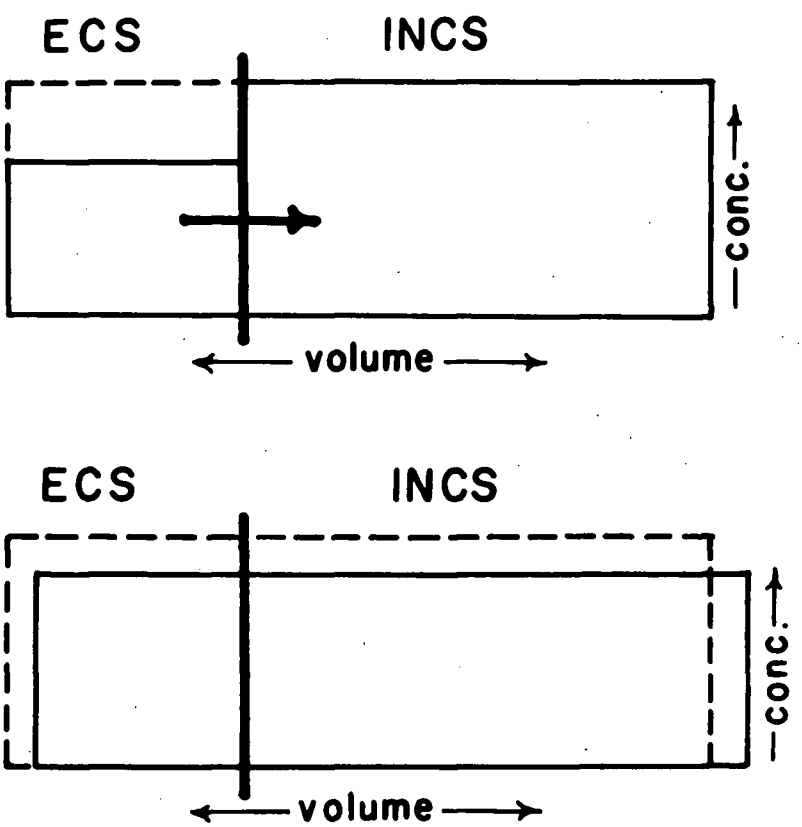
4a. Overhydration: A pure influx of water without salt depletion decreases the concentration of the extracellular fluid, and water shifts into the cells. The volume of the extracellular space is increased.

4b. Salt depletion: Loss of electrolytes with no change in total fluid volume results in shift of extracellular fluid into the cells. The volume of the extracellular space is decreased.

a.


ECS
INCS
conc.
volume
ECS
INCS
conc.
volume

b.


ECS
INCS
conc.
volume
ECS
INCS
conc.
volume

of solutes - electrolytes and proteins - that produce this pressure is different in the two phases. The influx of water lowers the osmotic pressure in the extracellular space. Water then freely passes the cellular membranes to the region of higher osmotic pressure, the intracellular space, thereby causing a swelling of the latter. The result is an increase in volume and weight and a decrease in concentration of both fluid phases (Fig. 4a).

The second major osmotic problem of fresh water flounder is the loss of salts. Pure salt depletion reduces the concentration of the extracellular phase and induces a movement of extracellular water into the cells (Fig. 4b). In this case the volume of the extracellular space decreases while the intracellular volume is increased. Thus, either water excess or salt depletion causes a swelling of the cells, though alterations of the extracellular fluid volume differs in each case. It is evident that concentration disturbance cannot be dissociated from volume disturbances, since a change in one affects the other. A fresh water flounder is regulating against both disturbances simultaneously - a superimposition of over-hydration on salt depletion. In osmotic failure in fresh water, the breakdown appears to affect the active transport of solutes - both the uptake of ions by the gills and the reabsorption of electrolytes from the glomerular filtrate by the renal tubules. With the drop in osmolarity of the plasma and interstitial fluids, water moves into the cells, causing the marked edema noted in flounder starved for long periods. The extracellular volume, however, decreases as water shifts to the cells and produces a drop in plasma volume with a decline in cardiac output and blood pressure. These cardiovascular changes were readily apparent in moribund fresh water flounder and at times in otherwise healthy flounder. With circulatory failure, renal plasma flow and glomerular filtration diminish so that even osmotic water which continues to enter the body cannot be eliminated. This vastly intensifies the problem and total osmotic collapse comes quickly.

IV

THE EFFECT OF ENVIRONMENTAL SALINITY ON THE OXYGEN CONSUMPTION OF FLATFISH

Osmotic independence of living organisms appears to have originated a number of times during animal evolution and radiated along several lines resulting in the appearance of rather diverse regulatory mechanisms to solve osmotic problems encountered in nature. Because of such diversity, it is not surprising that investigations of energy output associated with osmotic work in different groups of aquatic animals have resulted in a seemingly irreconcilable array of experimental findings. Table II presents a summary of the literature on the subject of the influence of salinity on oxygen consumption of fishes. A study of the table will convince the reader that it is not possible to fit all of these findings, some of which seem contradictory, together into one picture. In addition, the variety of techniques employed contribute to the inadequacy of the results for comparison with one another. In one case (Keys) standard metabolism was measured by the constant flow technique, but the usual method was simply to place the fish in a closed container for a measured period of time then remove it and determine the amount of dissolved oxygen consumed from the water (e.g. Busnel, Cordier and Leblanc, Cordier and Maurice, Graetz, Henschel, Raffy). Wohlschlag measured active metabolism in a closed circular plastic container. Leiner used the Warburg apparatus for his measurements of sea horse respiration.

Deviations in metabolic rate as affected by salinity have not always been accepted as indicative of altered energy demands for osmotic work. Schlieper (1929, 1935) in particular, has proposed alternate theories to account for respiratory changes in marine animals, primarily invertebrates, exposed to variations in environmental salinity. In general, the theories are attempts to explain the frequently observed increase in metabolic rate of marine invertebrates

Table II. The influence of salinity on oxygen consumption of teleost fish as reported in the literature.

Species	Habitat	Regulatory capacity	Salinity effect	Author
Salmo iridaeus & Salmo salar (eggs & alevins)	fresh water	hyper-osmotic regulator	Salinity change has no effect. During growth O_2 consumption increases more rapidly in fresh water.	Busnel, et.al., 1946
Tinca vulgaris	fresh water	stenohaline hyper-osmotic regulator	Transfer to salinities of 10 to 15°/oo results in gradual fall in O_2 consumption to death, more rapid in higher concentrations. Death apparently by asphyxiation.	Cordier and Maurice, 1957
Tinca tinca	fresh water	stenohaline hyper-osmotic regulator	Increased salinity caused fall in in O_2 consumption followed by death.	Raffy, 1932, 1933.
Carassius carassius	fresh water	stenohaline hyper-osmotic regulator	In salinities up to isotonicity, O_2 consumption increased. In hypertonic media, O_2 consumption decreased to 60% of normal.	Veselov, 1949
Anguilla vulgaris (juvenile)	migrating sea to fresh water	euryhaline	O_2 consumption less in fresh water.	Raffy and Fontaine, 1930.
Anguilla vulgaris (adult)	migrating fresh to sea water	euryhaline	O_2 consumption less in fresh water.	Raffy, 1933
Fundulus parvipinnis	marine	euryhaline	Abrupt transfer to fresh water causes decrease in O_2 consumption. Returns to normal after fresh water acclimation.	Keys, 1931

Table II. (continued).

Gasterosteus	fresh water	euryhaline	O_2 consumption 20 - 30% higher in fresh water than isotonic sea water. No further decrease with increase in salinity.	Graetz, 1931
Coregonus sardinella	fresh water and marine migratory	euryhaline	Marine (migratory) forms have higher metabolic rates than fresh water forms	Wohlschlag, 1957
Scorpaena porcus	marine	stenohaline hyper-osmotic regulator	Transfer to fresh water causes immediate drop in respiration to half normal sea water rate, then followed decline to death.	Cordier and Leblanc, 1955
Scyllium catulus	marine	stenohaline hyper-osmotic regulator	Transfer to dilute sea water or fresh water causes O_2 consumption drop followed by death.	Raffy, 1932, 1933
Sargus rondeletei	marine	stenohaline hyper-osmotic regulator	Transferred to dilute sea water, O_2 consumption at first rises, then drops suddenly followed by death.	Raffy, 1933
Pleuronectes platessa (juvenile)	marine	hypo-osmotic regulator	Transfer to dilute sea water and fresh water had no significant effect on oxygen consumption.	Raffy, 1955
Pleuronectes platessa (adult)	marine	hypo-osmotic regulator	Low salinities cause increased O_2 consumption.	Henschel, 1936
Hippocampus	marine	stenohaline hypo-osmotic regulator	Transfer to dilute sea water causes transitory increase in respiration. Large dilution causes continual fall. In concentrated sea water, O_2 consumption rises briefly then returns to normal. If to saline, respiration falls continually.	Leiner, 1938

moved into brackish or fresh water. His first theory (1929), which he himself subsequently refuted, assumed that oxygen demands were less in high salinities because carbon dioxide could be eliminated more efficiently in salt than fresh water, thus reducing the carbon dioxide content of the blood. In the second theory (1935) Schlieper proposed that the oxygen content of tissues was directly related to their water content. In low salt concentrations water would flood and swell the cells, thereby increasing their surface area and facilitating the exchange of respiratory gases. Arguments have been raised against the theory (e.g. Kuenen, 1939) but it does offer a convenient explanation for the oft-observed increase in respiration of marine poikilosmotic animals introduced into low salinities.

It is paramount in any study of the energetics of osmotic regulation that a full appreciation is given the osmotic capabilities of the species studied. Measurements of oxygen consumption of a fish subjected to a salinity beyond that which it can indefinitely tolerate have doubtful significance, because the observed changes may be of a pathological nature. Even in euryhaline forms, a change in the energy requirements concomitant with salinity changes must be interpreted with caution. Transitory increases in oxygen consumption usually are measured when a fish is introduced into a new salinity and are the result of the stress imposed. It is important, therefore, that the animal be adapted to the experimental salinities before definite determinations of oxygen consumption are made. In the published literature on the effect of salinity on the oxygen consumption of fishes, it would appear that inadequate attention has been paid to either proper salinity adaptation or the osmotic capacity of the species.

The indirect determination of metabolic rate in fishes as measured by the consumption of dissolved oxygen needs to be carried out with certain precautions in mind. Fortunately the problems of such measurement have recently become appreciated and the unrecognized pitfalls encountered by the earlier

workers can now be largely avoided. The most important considerations, as discussed in a recent review by Fry (1957) are:

1) Body size.

2) Experimental temperature and thermal history.

3) Oxygen and carbon dioxide tensions.

4) Seasonal influences.

5) Diurnal variations.

6) Activity.

7) Nutritional state.

8) Sex and sexual maturity.

9) Environmental salinity.

In this investigation the attempt has been made to control, eliminate, or take into account all of these influences, leaving one variable, salinity, to be experimentally altered. Because the effect of size on the metabolic rate–thyroid activity interrelationship formed an important part of the investigation, a fairly large size range of flounder (about 4 to 300 grams) was used. This was as wide a range as the sensitivity of the method and the capacity of the apparatus would allow. Among the important advantages of using a wide range of sizes are that the data lend themselves well to statistical treatment and yield a great deal more information regarding the influences of the experimental variable, in this case salinity. In addition, important quantitative differences in the effect of salinity between small and large fish may come to light.

In this discussion, the terms metabolism, total metabolism, oxygen consumption, oxygen uptake and respiration will refer to "oxygen consumption <u>per fish</u> per hour". The terms metabolic rate, respiratory rate, respiratory intensity, weight-specific oxygen consumption and rate of oxygen consumption will mean "oxygen consumption <u>per gram body weight</u> per hour."

A. DETERMINATION OF STANDARD METABOLIC RATE

1. Apparatus.

Standard metabolism was measured in an apparatus utilizing the constant flow principle described by Keys (1930). This method is presently gaining wide favor for oxygen consumption determinations in fish (Job, 1955; Shepard, 1955; Fry, 1957). The fish is placed in a chamber through which water continuously flows and the oxygen consumption determined by analysing the oxygen content of the inflowing and outflowing water and the rate of water flow through the chamber. Because a variety of experimental salinities was needed, the apparatus used here was modified so that the water of the desired salinity could be continuously recirculated. The respiratory chambers, containing individual fish, were submerged side by side in an insulated trough, 264 cm. long by 25 cm. wide. Water from an elevated 120 liter barrel entered the trough at one end through a constant-level bottle and left by an overflow at the other. Overflow water and outflowing water from the respirometers was collected in a 100 liter tank below and pumped to the reservoir above. The water level in the reservoir was maintained by a float and mercury switch which controlled the operation of the pump below.

Water was cooled in the collecting tank with a series of glass cooling tubes through which cold fresh water flowed, and then held thermostatically at the desired temperature. During an entire experimental run (about 24 hours) the temperature varied less than 0.4°C. and less than 0.1°C. from one end of the respiration trough to the other at any one time. The circulating water was vigorously aerated in the collecting tank and repeated analyses showed that the oxygen tension in the trough was always at air saturation.

Because flatfish are laterally-compressed they present a special problem in the selection of a proper respiration chamber. The clear Lucite refrigerator boxes manufactured by Tri-State Plastics, Louisville, Kentucky, proved to be admirably suited for this purpose. From a variety of obtainable sizes, four were

selected which would accomodate a size range of flounder up to 300 grams and lemon sole to 200 grams. The dimensions and capacities of the chambers were: 26.3 x 19 x 10.2 mm. (5.1 liters), 18.6 x 13 x 10.2 mm. (2.47 liters), 16.7 x 11.8 x 6.7 mm. (1.32 liters), 11.8 x 8.2 x 6.6 mm. (0.64 liters). The boxes were drilled on one end and a two-hole rubber stopper was fitted with inflow and outflow tubes. Water entering the respirometer through the longer inflow tube passed the length of the box to the opposite end. The outflow tube was short and was connected to a length of tygon tubing which syphoned the effluent over the edge of the trough and into a sample bottle (30 ml. glass-stoppered Erlenmeyer flasks). Rate of water flow through each respirometer was controlled by raising or lowering the sample bottle with a number of thin plywood shims. This method was found superior to regulating the flow with a clamp, which tended to trap air bubbles or excreta, resulting in tube blockage. Just prior to collecting water samples for oxygen analysis, flow rates were measured by collecting the overflows from the sample bottles in graduate cylinders. The error between duplicate flow measurements was usually less than one percent for the large respirometers with a fast flow and up to 3 percent for the small respirometers with a relatively slow flow. A view of part of the apparatus is shown in Figure 5.

The respirometer used corresponded roughly to the size of fish, with a volume of water in the chamber 15 to 100 times the volume of fish. In accord with Geyer and Mann (1939), who found that a respiration chamber with a volume smaller than 10 times that of the fish caused over-excitement of Perca fluviatilis, it was noted that a chamber at least 10 times the volume of flounder was necessary to avoid heightened respiration. A layer of washed, screened sand was placed in the bottom of each respirometer. The flatfish usually buried themselves in the sand, leaving only the upper surface of the head and operculum exposed.

Figure 5. A view of part of the apparatus for standard metabolism measurements. Shown from above is the insulated trough containing individual respirometers. Tygon tubing leads from each respirameter to a sampling flask set in a small plastic dish and arranged such that the overflow can be collected in graduate cylinders below for flow measurements.

30
31
14
15
17
A

2. Chemical Analysis

Dissolved oxygen was determined by a semi-micro modification of Winkler's iodometric technique, observing the precautions discussed by Ohle (1953). A 20 ml. aliquot part of the sample was titrated, using a microburette and a dilute (.01N) thiosulphate solution. The error in repeated titrations of one fixed sample was less than one percent.

3. Experimental Procedure

A number of workers (e.g. Keys, 1930; Wells, 1932; and Black, Fry, and Scott, 1939) have demonstrated that the handling of fish necessary to place them in the experimental chamber affects a greatly heightened oxygen consumption which subsides only gradually to a standard rate several hours later. For this reason it is necessary to maintain the fish several hours under controlled conditions before the first samples are drawn. Fish were introduced into the respirometers the evening before the day that measurements of metabolic rate were made. They thus had a period of 18 to 22 hours in the chambers under constant experimental conditions (darkness, quiet, and constant temperature, salinity and water flow).

Another influence which tends to supplement the physiological variation in metabolic rate already present is that of endogenous 24 hour cycles in respiratory intensity. Endogenous cycles have been found in fish respiration by a number of workers (e.g. Clausen, 1936; Spoor, 1946 and Higgenbothan, 1947). Pilot experiments showed the presence of such a diurnal rhythm in starry flounder, with consumption high in the morning, decreasing measurably until about 1:00 p.m., and leveling off during the remainder of the afternoon until about 6 p.m., when it increased sharply. To minimize this influence and ensure basal conditions all samples were collected during the afternoon.

The oxygen consumption of 12 to 18 fish was determined during each experimental run. After adjusting the water in the apparatus to the desired ex-

perimental salinity and temperature, the fish were transferred with a minimum of handling from the holding tank to the respirometers. The respirometer tops were then sealed with a heavy, non-toxic grease (Nevastane, Heavy X, Keystone Co.). When all the fish were in place, the trough was covered and flow rates adjusted so that under conditions of standard metabolism the concentration of dissolved oxygen in the water leaving the respirometers would be between 70 and 85 percent air saturation. At 1 and 3 p.m. of the following afternoon, flow rates were measured and samples of outflowing water from each respirometer were immediately collected, fixed and analyzed for dissolved oxygen. A third sample was analyzed if the first two differed in excess of about 5 percent. At these same times three samples were drawn for oxygen content analyses of the water at both ends and in the center of the trough (inflowing water). After completion of the experiment, the fish were removed from respirometers, weighed (wet weight with bodies blotted) and returned to the holding tanks.

4. Statistical Procedures

Rate of oxygen consumption was plotted as a function of body weight (weight-specific) in a double logarithmic coordinate system. This method was selected over the other common mode of presentation, i.e., logarithm of total oxygen consumption against logarithm of body weight, because it graphically emphasizes the weight dependence of respiratory intensity. When plotted on a double logarithm grid, weight-specific oxygen consumption of animals over an adequate size range depicts a straight or nearly straight line with a negative slope. Regression of total oxygen consumption against body weight is of the form:

$$O_2 = aW^b$$

or

$$\log O_2 = \log a + b \log W$$

where O_2 is total oxygen consumption of the fish in mg O_2/gm./hr., W is body

weight in grams, a the intercept, and b the slope of the line. Since rate of oxygen consumption was preferred for presentation here, the equation was transformed to weight-specific by dividing by W.

Thus:

$$\frac{O_2}{W} = aW^{b-1}$$

or:

$$\log O_2 - \log W = \log a + b \log W - \log W$$

The problem here was to test whether statistically significant differences existed between two regression lines, representing the metabolic rates of flatfish measured in two different salinities. Usually oxygen consumptions of 12 to 24 fish were determined for each experimented salinity during a test series and often considerable variability existed around each calculated regression line. Data of this nature are readily adapted to statistical treatment by analysis of covariance. The procedure was to test by analysis of covariance the null hypothesis that no true differences existed in the effect of two salinities on oxygen consumption, i.e., that the two regression lines could be represented just as well by a single regression line. To eliminate negative log Y values in the statistical analysis, oxygen consumption rates were multiplied by 100 before converting to common 5-place logarithms. A standard method of covariance analysis outlined by Ostle (1954) for a randomized design was followed.

B. RESULTS

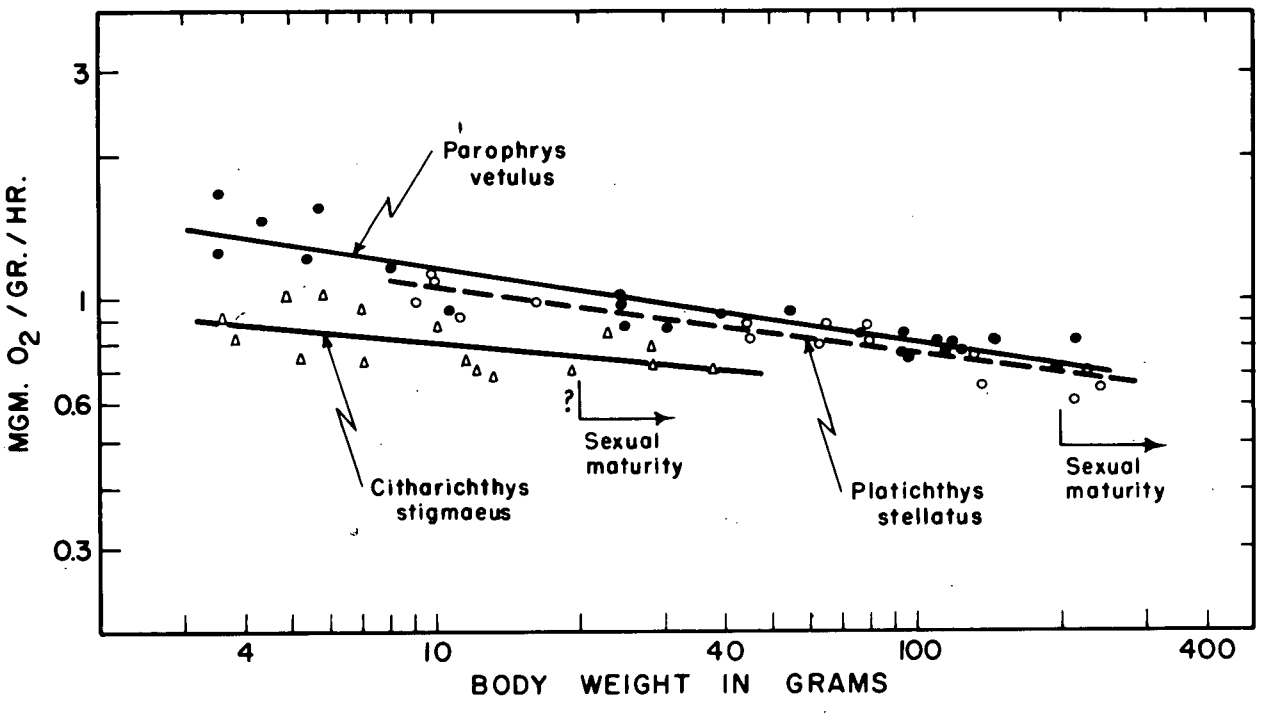
1. Standard Metabolic Rates of Starry Flounder, Lemon Sole and Speckled Sand Dab

a. Interspecific comparison

In Figure 6a a graphic comparison is made of standard metabolic rates of flounder, sole and sand dab in sea water of 25°/oo. The data are given in Table III. Measurements were made at a temperature of 15°C. and at a time when

Figure 6a. Standard metabolic rate of Platichthys stellatus (open circles), Parophrys vetulus (closed circles) and Citharichthys stigmaeus (triangles) in sea water of 20–25 °/oo. Lines are fitted by the method of least squares. Estimated body weight at the time of first maturity is indicated as approximately 20 grams for the sand dab and 200 grams for the flounder and sole. Experimental temperature 14–15°C. Data of Table III.

Figure 6b. Standard metabolic rate of winter Platichthys stellatus showing the departure from the linear log weight-log rate relationship seen in summer flounder. Line fitted by eye. Experimental temperature 10°c , salinity 24.7–26.0 °/oo.


3
1
0.6
0.3
MGM. O_2 / GR. / HR.
Parophrys vetulus
Citharichthys stigmaeus
?
Sexual maturity
Platichthys stellatus
Sexual maturity
4
10
40
100
400
BODY WEIGHT IN GRAMS

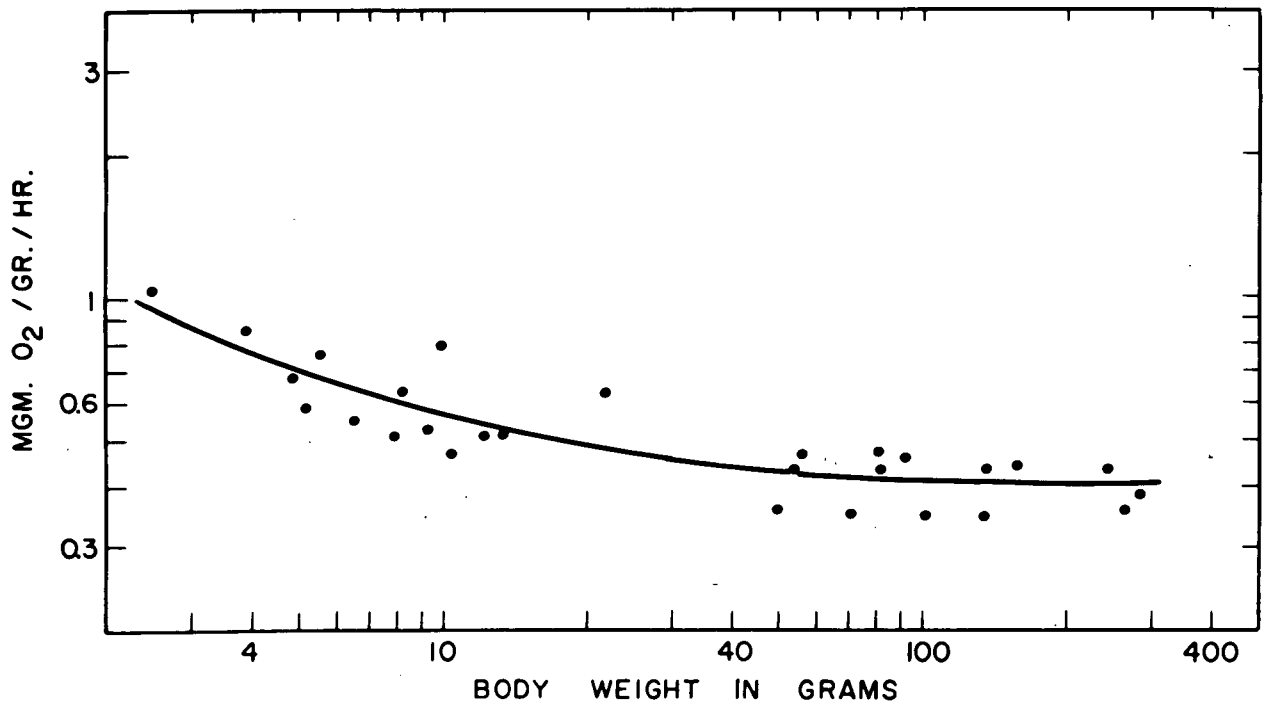

3
1
0.6
0.3
MGM. O_2 / GR. / HR.
4
10
40
100
400
BODY WEIGHT IN GRAMS

Table III. Standard metabolic rates of Platichthys stellatus, Parophrys vetulus and Citharichthys stigmeaus in sea water. X = body weight in grams, Y = rate of oxygen consumption in mgm. O_2/gm./hr. (average of 2-3 determinations).

Parophrys vetulus		Platichthys stellatus		Citharichthys stigmaeus	
X	Y	X	Y	X	Y
3.5	.123	9.0	.099	3.6	.092
3.5	.165	9.9	.114	3.8	.082
4.3	.145	10.1	.109	4.9	.102
5.3	.121	11.3	.092	5.2	.075
5.7	.155	16.0	.100	5.8	.103
8.0	.117	45.6	.084	7.0	.095
10.8	.094	45.6	.080	7.1	.074
24.1	.099	62.4	.081	10.2	.088
24.7	.101	65.6	.089	11.6	.075
24.7	.089	78.0	.084	12.3	.071
29.8	.086	79.0	.088	13.1	.070
39.1	.092	132.3	.077	19.1	.071
53.8	.095	139.1	.064	22.9	.086
77.7	.085	212.2	.062	28.1	.080
92.1	.084	223.6	.071	28.3	.074
93.4	.077	245.6	.065	38.0	.076
97.5	.075				
110.7	.082				
113.0	.075				
117.8	.080				
124.3	.077				
143.8	.083				
191.6	.071				
217.0	.082				
Exp. temp. 15.3±.2°C.		Exp. temp. 15.0±.1°C.		Exp. temp. 14.0±.2°C.	
Exp. sal. 20.0‰		Exp. sal. 22.8‰		Exp. sal. 24.4‰	
June 30, 1956		May 13, 1957		July 5, 1957	
Slope = -.158		Slope = -.141		Slope = -.095	

all species were in a similar nutritional state (3–4 days of fasting).

Weight-specific oxygen consumption of flounder and sole are essentially the same throughout the size range examined but greater than the metabolic rate of sand dab at an equivalent body size. In addition, there exists a more rapid depression of metabolic rate with increasing size of flounder and sole (b = -.141 and -.158 respectively) than of sand dab (b = -.095). However, it is important to recognize that one is dealing with an inter-specific comparison of fish that differ markedly with respect to ultimate size attained and size at sexual maturation. Included in Figure 6a is an indication of the size of each species at first maturity. In both starry flounder and lemon sole, males usually reach maturity a year ahead of females (a general phenomena among fishes) - roughly at 200–250 grams body weight (Orcutt, 1950; Ketchum, 1947, 1956). Starry flounder grow very large, in excess of 9,000 grams and lemon sole to perhaps 3,000 grams. The sand dab, on the other hand, is a small species. Sexually mature females of 20 grams were occasionally found, and the largest individuals of the species rarely exceed 50 grams. Hence, with respect to the ultimate sizes of three species, it is clear that altogether different periods of ontogeny are represented in the metabolism curves.

Figure 6a shows that the respiratory intensity of sand dab at maturation (about .075 mgm. O_2/gm./hr.) is essentially the same as that of sole and starry flounder at maturation (about .070 mgm. O_2/gm./hr.). Whether or not the metabolic rate of these species is actually more dependent on the rate of growth and physiological age than upon body size, per se, will have to await further experimentation.

b. Significance of the slope of regression of metabolic rate

The relationship of metabolism to body size as expressed by the formulae

$$O_2 = a\text{Weight}^b$$

implicates a weight or allometric dependence of oxygen consumption of animals. According to the surface concept, established by Rubner (1883), metabolism is a 2/3 power of body weight. Plotting oxygen consumption as a function of body weight on a double logarithmic grid, the slope of a surface proportional curve is 0.67 as opposed to a slope of 1.0 for weight proportionality. Plotted as weight-specific metabolism as expressed by:

$$\frac{O_2}{W} = aW^{(b-1)}$$

the slopes of regression for weight and surface proportionalities are 0 and -0.33 respectively.

Slopes of weight-specific metabolism of flatfish studied here invariably gave values falling between weight and surface proportionality. Considerable intraspecific variation in (b-1) was witnessed which could not always be attributed to any specific environmental influence. However, as will be shown, important shifts in slope occurred concomitant with transfers of flounder to a changed osmotic gradient.

Considerable significance has been attached to the exponent b for a species in spite of recent reviews (Zeuthen, 1947, 1953, 1955) pointing out the wide range of values that the slope, b, can take. Bertelanffy (1951, 1957) has categorized a large number of animals, including fish, into "metabolic types" depending on the proportionality (surface, weight or intermediate) of metabolism. Notwithstanding numerous examples to the contrary (Fry, 1957) Bertalanffy has lumped fish as a group into one "metabolic type" - surface proportionality. The assumption made but not stated is that the slope neither changes during the ontogeny of the species nor is influenced by physiological alterations affected by environmental stress. Neither of these assumptions is tenable on the basis of experimental evidence.

Zeuthen (1953) has presented several examples of animals which show significant changes in metabolic rate during their ontogeny. Fishes form no

exception. Shamardina (1954) found that metabolic rate of pike increased with growth of larvae, then inflected sharply and fell during post-larval, juvenile and adult growth. The same author sites similar findings of three other Russian workers: Bezler woking with carp and bream, Korzhuiev with sturgeon and Privolnev with salmon. Lindroth (1942) and Zeuthen (1947) also report significant changes in slope during growth of fish. Therefore, it is impossible to neglect ontogenetic changes in the percentage decrease in metabolic rate with increasing body size of fish.

Usually no ontogenetic change in slope was apparent in the present studies on flounder and sole. All of the spring and summer determinations yielded data that conformed to a straight line on logarithmic paper. However, data collected on winter flounder measured at 10°C. strongly suggest a percentage increase in slope towards weight proportionality with increasing body size. These data are shown in Figure 6b. Why such an effect should appear in the winter (10°C.) but not in the summer (15°C.) is difficult to understand, but it is not inconceivable that thepercentage increase in slope reflects heightened metabolic demands with the approach of sexual maturity and the spawning season (December through March). For purposes of statistical comparison of data collected with different salinity treatments, the points were assumed to conform to a linear log weight-log rate relationship with no change in slope. The data in Figure 6b, for instance, is fitted with a straight line in Figure 12b for convenience of treatment comparisons.

Sometimes changes in slope were noted in comparing weight-rate curves obtained from flounder measured at the same salinity and temperature but at different times of the year. For example, the slopes of the metabolic rate of flounder in sea water measured on May 13 and June 29 at the same experimental temperature (15°C.) were -0.141 (Fig. 13a) and -0.311 (Fig. 11a) respectively. The results imply seasonal effects. Though seasonal influences or cycles were

not a part of this work and were not studied, the few observations made show the need to account for seasonal effect before placing any significance in the slope of regression of metabolic rate.

2. Diurnal Rhythm in the Metabolic Rate of Starry Flounder

In the pilot experiments an endogenous cycle was indicated in the metabolic rate of starry flounder. Successive samples drawn for dissolved oxygen analysis during the morning hours showed a gradual decrease in oxygen consumption until early afternoon, when oxygen consumption appeared to level off. Accordingly, for all subsequent experiments, samples for snalysis were collected in the afternoon when fluctuations in metabolic rate were minimal. It is emphasized that the fish were under controlled experimental conditions in excess of 12 hours, even for the morning measurements, certainly an adequate delay to ensure that the high morning values were not the result of the stimulation of handling in placing the flounder in the respirometers.

To assess the complete diurnal activity cycle, 14 flounder of all sizes, from 6.7 to 225 grams, were simultaneously carried through one 24 hour period. After the initial 18 hour delay, oxygen consumption measurements were begun and continued for 24 hours, with measurements every 3 hours. During this period the fish were completely protected from external stimuli such as light, noise and vibration. The temperature varied 0.1°C. The fish had been acclimated to temperature (15°C.) and salinity (26.5°/oo), conditions closely approximating those in the environment from which the fish were captured at that time of year (late September). They were starved four days prior to the experiment.

The results of the experiment are summarized in Table IV. In Figure 7 the weight-specific oxygen consumptions of the 14 flounder are plotted for each of the 8 sampling periods during the 24 hours and lines of best fit calculated by the method of least squares. The data were tested by analysis of covariance

Figure 7. Metabolic rates of 14 Platichthys stellatus measured at 3-hour intervals over one 24 hour period. Each point represents one determination for a fish at the indicated time of day. Lines are fitted by the method of least squares. Experimental temperature 15.0°C., salinity 26.6 °/oo. September 26-28, 1957. Data of Table IV.

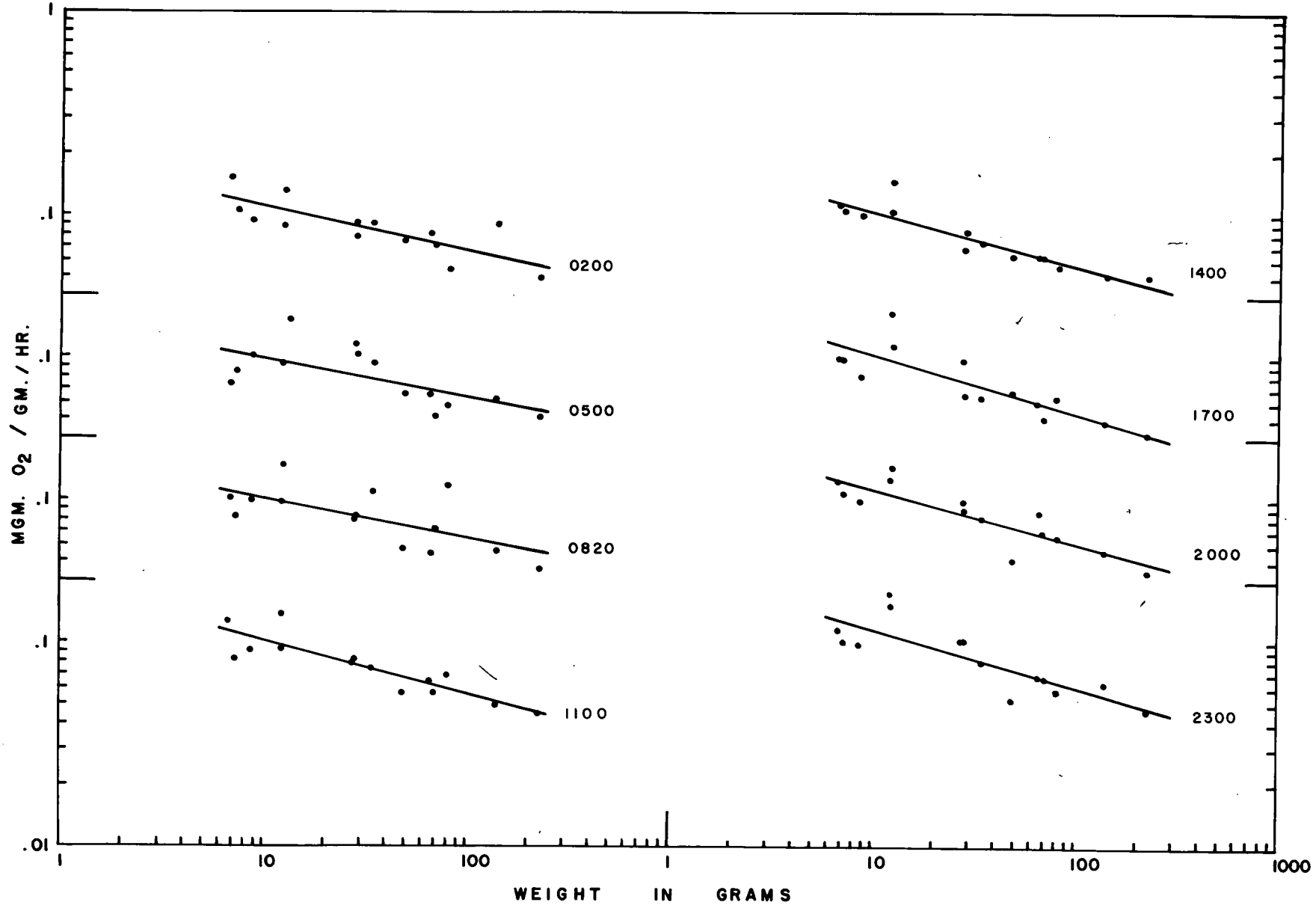

MGM. O_2 / GM. / HR.
1
.1
.01
1
10
100
1
10
100
1000
WEIGHT IN GRAMS
0200
0500
0820
1100
1400
1700
2000
2300

Table IV. Diurnal variation of metabolic rate in Platichthys stellatus. Body weights and rates of oxygen consumption in mgm. O_2/gm./hr. of 14 flounder followed through one 24 hour period (September 25-26, 1957). Determinations made at 3 hour intervals.

	Time of Day							
	0200	0500	0820	1100	1400	1710	2000	2300
Body Weight	Rate of Oxygen Consumption							
6.7	.152	.0735	.101	.127	.113	.0995	.125	.117
7.1	.104	.085	.082	.100	.105	.0995	.109	.102
8.6	.093	.102	.0995	.091	.100	.081	.0995	.0995
12.1	.088	.093	.0975	.093	.105	.167	.129	.177
13.3	.131	.152	.149	.139	.148	.115	.147	.153
27.7	.0775	.115	.080	.066	.069	.098	.0995	.104
28.1	.091	.101	.082	.083	.084	.076	.0905	.104
34.0	.09	.0925	.11	.075	.075	.0645	.083	.081
48.0	.0745	.065	.0565	.0565	.064	.069	.0515	.053
65.0	.081	.065	.054	.065	.065	.061	.088	.069
69.0	.071	.068	.072	.057	.064	.051	.070	.069
80.0	.054	.058	.118	.070	.057	.0645	.067	.058
138.5	.0895	.0625	.0555	.0495	.0517	.039	.0572	.063
225.0	.0485	.051	.046	.045	.051	.0425	.0457	.0465

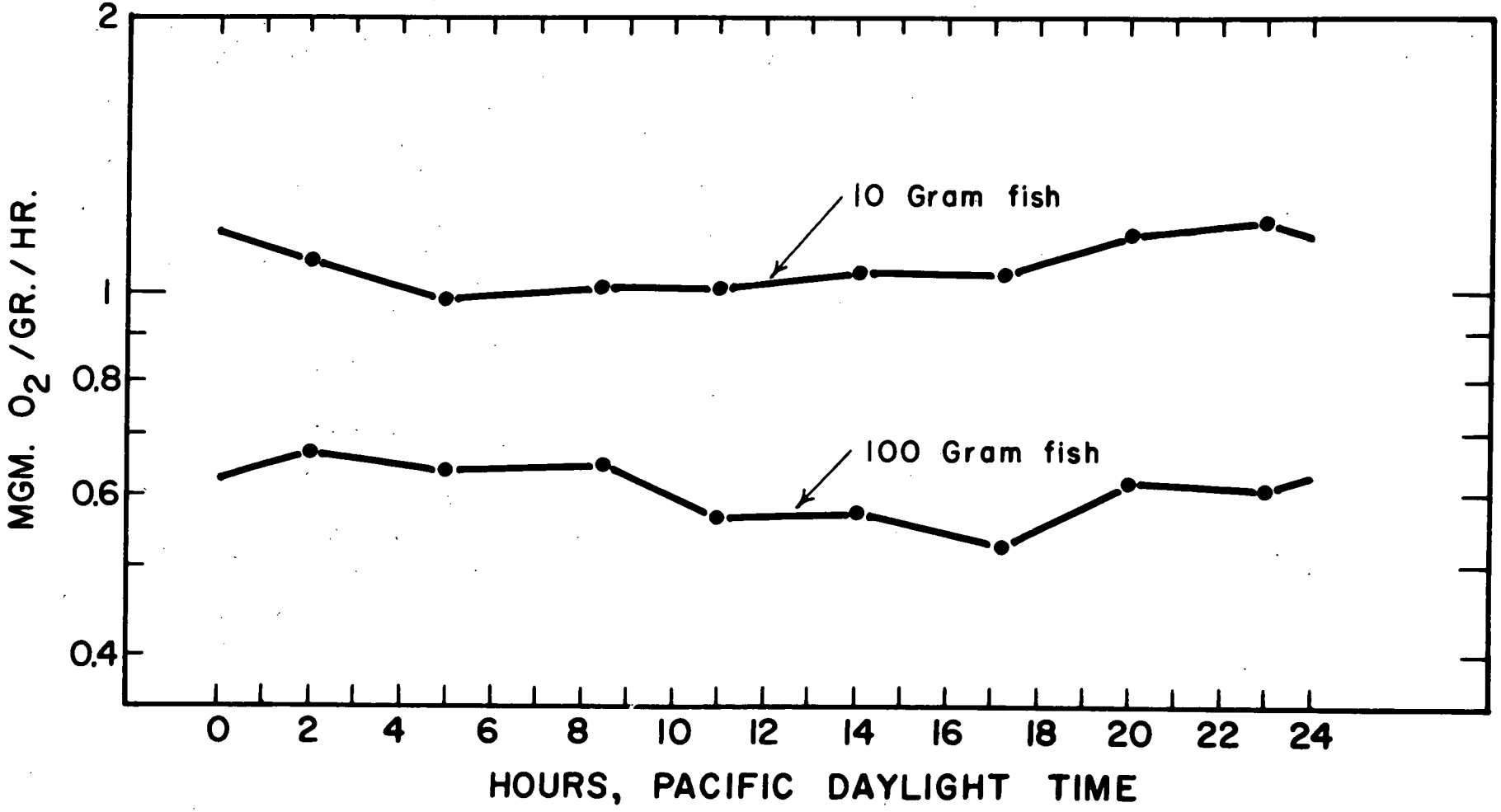
Exp. temp. 15.0-15.1°C.
Exp. sal. 26.6°/oo
Days unfed = 4

to determine whether true differences existed between the 8 regression lines. The hypothesis that there are no differences between effect of the time periods is rejected at the 1% probability level (Appendix - table I), indicating that a true diurnal rhythm is present.

Considerable variability is present around the regression line, the small flounder in particular showing rather wide departures from the mean. Part of this variability is due to the error in the method because it was possible to analyze only one oxygen sample for each fish at each time period, whereas at least two and often three samples were collected for each fish in the salinity effect experiments and the results averaged. But most of the variation is due to true physiological differences in the metabolic rates of the flounder themselves. A close examination of Figure 7 shows that certain of the flounder in particular hold definite positions above or below the regression line. The 12.3 gram flounder, for instance, would appear to have an inherently high total metabolism, the 7.1 and 81.6 gram animals, inherently low metabolic rates. This sort of intrinsic variation is well known in lower vertebrates, is normal but troublesome, and necessitates using a rather large sample size for proper statistical treatment.

Figure 8 represents a summary of the experiment. From each regression line two points were taken, one representing a 10 gram, the other a 100 gram flounder, and plotted on a semi-logarithmic grid. The cycle is illustrated best by the lower plot (for 100 gram flounder) since less variability existed in the large than in the small fish. Oxygen consumption is highest at night. With the approach of daylight hours (though these experimental animals were maintained in darkness throughout) the rate of oxygen consumption declines and, at least for the large fish, reaches a low point in late afternoon before increasing sharply in the evening. The small flounder did not show a similar decline during the daylight hours but it is possible that any true changes were masked by the large variability of the small fish.

Figure 8. Diurnal variations in metabolic rate of Platichthys stellatus. The "10 gram fish" and "100 gram fish" rates are derived from the points in Figure 7 where the lines of best fit cross the 10 gram and 100 gram lines of each of the 8 time periods.


2
1
0.8
0.6
0.4
MGM. O_2 /GR./HR.
10 Gram fish
100 Gram fish
0
2
4
6
8
10
12
14
16
18
20
22
24
HOURS, PACIFIC DAYLIGHT TIME

3. Effect of Starvation on the Standard Metabolic Rate of Starry Flounder

Two experiments were performed to assess the effect of starvation on the rate of oxygen consumption. As with the previous experiment on diurnal rhythm, this experiment was not designed in any way as a definitive study of caloric starvation effects on metabolic rate but rather to evaluate the influence of this variable on the salinity effect experiments to be presented in the next section. Both spring (May) and winter (December) fish were studied.

Spring experiment

The oxygen consumption of a selected size range of flounder was determined on the fourth, seventh and twentieth days after collection. During this period, the flounder were held unfed under controlled conditions of temperature (15°C.) and salinity (25±2°/oo). As with all the metabolism experiments, the animals were introduced into the respirometers 20 hours before water analyses were begun.

The results tabulated in Table V are plotted individually in Figure 9. The three starvation periods were statistically treated by analysis of covariance two at a time to determine whether true decreases in oxygen consumption occurred. In both cases, the tests showed significant drops in standard metabolism (Appendix-table II).

Winter experiment

The temperature of the environmental locale of the winter fish collected in mid-December was 10.5°, thus the experiments were performed at 10°C. In other respects, experimental conditions were the same in both the winter and spring studies. Standard metabolic rate of a selected range of small to medium sized flounder was determined on thesecond and eleventh days of starvation and are given in Table VI and Figure 10a. The adjusted means of regression of respiratory rate on body weight were compared by covariance analysis (Appendix-table III) and the difference in the means found highly significant.

Figure 9. Standard metabolic rate of spring Platichthys stellatus starved 4, 7 and 20 days. Respiratory rates are fitted by the method of least squares. Experimental temperature 14.9 ±.2°C., salinity 22.8–26.4 °/oo. May, 1957. Data of Table V.

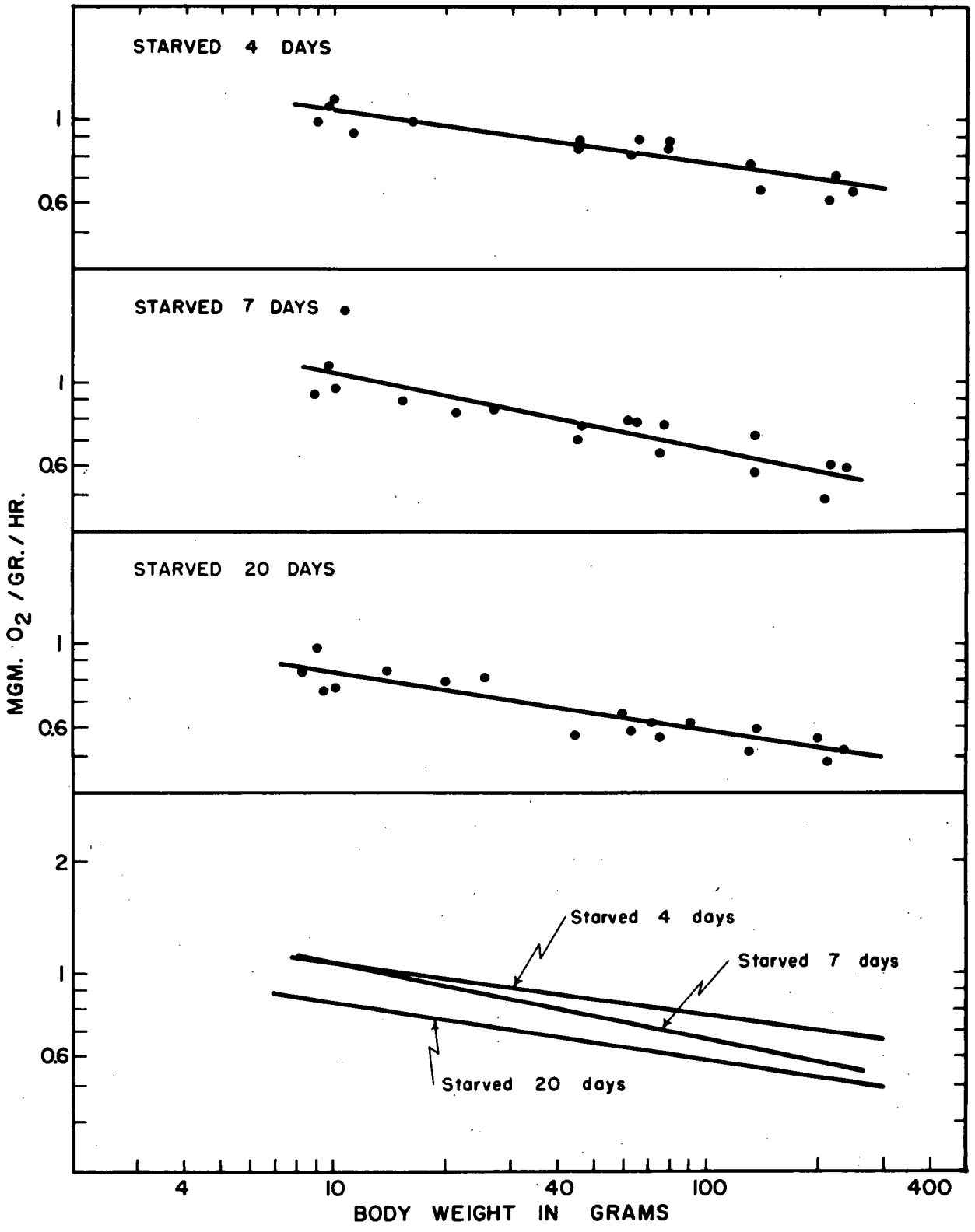
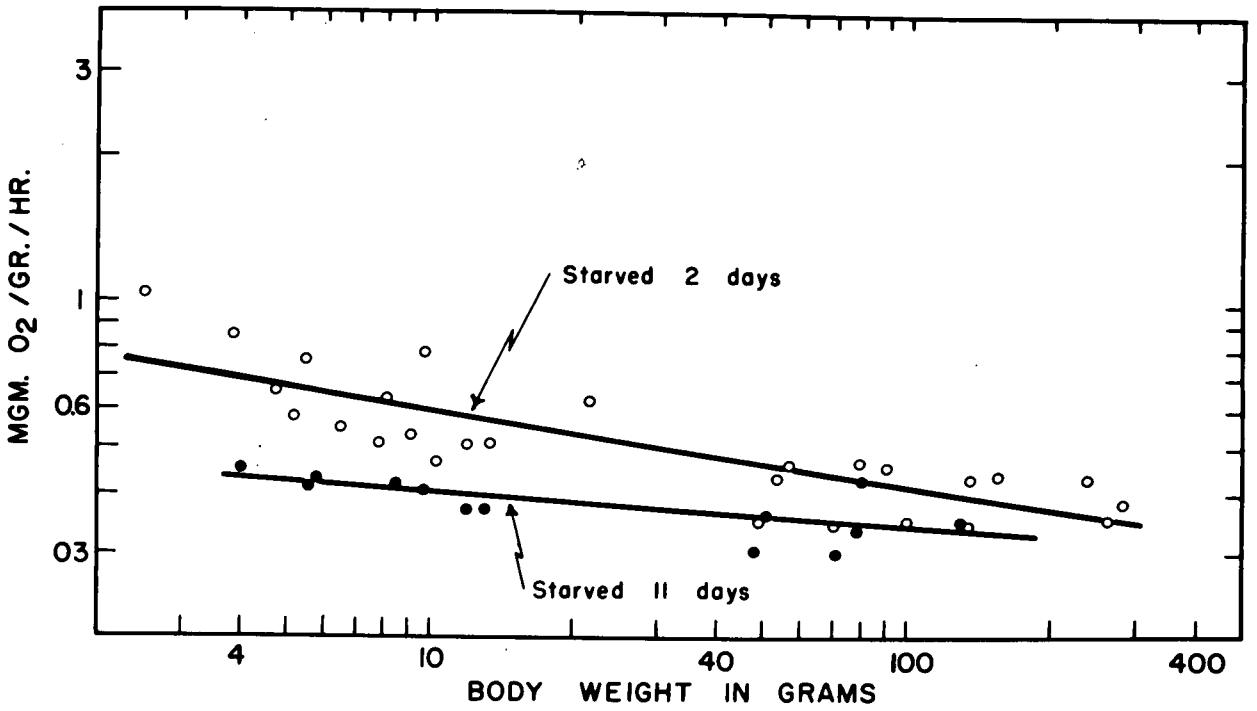

STARVED 4 DAYS
STARVED 7 DAYS
STARVED 20 DAYS
1
0.6
2
MGM. O2 / GR. / HR.
Starved 4 days
Starved 7 days
Starved 20 days
4
10
40
100
400
BODY WEIGHT IN GRAMS

Figure 10a. Standard metabolic rate of winter Platichthys stellatus starved 2 and 11 days. Respiratory rates are fitted by the method of least squares. Experimental temperature 10 ± 1°C., salinity 24.7 - 26.0 °/oo. December, 1956. Data of Table VI.

Figure 10b. Decrease in standard metabolic rate of Platichthys stellatus due to starvation. Points represent adjusted means of regression lines (adj. $\bar{Y}_i = \bar{Y}_i - b(\bar{X}_i - \bar{X})$). shown in Figures 9 and 10a.


3
1
0.6
0.3
MGM. O2 /GR./ HR.
Starved 2 days
Starved 11 days
4
10
40
100
400
BODY WEIGHT IN GRAMS

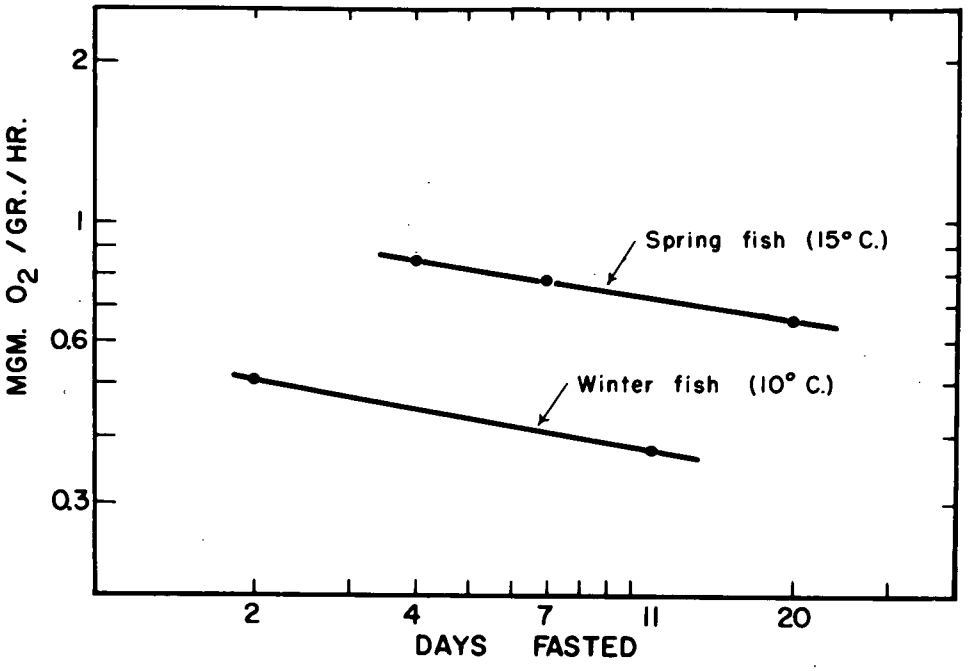

2
1
0.6
0.3
MGM. O2 /GR./ HR.
Spring fish (15° C.)
Winter fish (10° C.)
2
4
7
11
20
DAYS FASTED

Table V. Effect of starvation on the standard metabolic rate of summer Platichthys stellatus. X = body weight in grams, Y = rate of oxygen consumption in mgm. O_2/gm./hr. (average of 2-3 determinations).

Days Fasted					
4		7		20	
X	Y	X	Y	X	Y
9.0	.099	8.8	.093	8.2	.0844
9.9	.114	9.8	.111	9.0	.098
10.1	.109	10.0	.097	9.5	.075
11.3	.092	10.6	.146	10.3	.0762
16.0	.10	15.3	.089	14.0	.085
45.6	.0895	21.3	.0825	20.1	.080
45.6	.084	26.6	.085	25.6	.0825
62.4	.081	44.8	.071	44.8	.0575
65.6	.0893	45.7	.077	59.9	.0655
78.0	.0845	61.1	.079	62.4	.0577
79.0	.088	64.5	.079	71.6	.0615
132.3	.0765	75.0	.065	75.0	.0566
139.2	.0645	76.6	.0775	90.5	.062
212.1	.0618	131.7	.072	130.7	.0515
223.6	.071	131.8	.0575	137.3	.0595
245.6	.065	205.5	.049	98.8	.056
		215.7	.06	213.9	.048
		236.9	.0595	236.9	.052
Exp. temp. 15.0±.1°C.		Exp. temp. 14.8±.1°C.		Exp. temp. 14.9±.2°C.	
Exp. sal. 22.8°/oo		Exp. sal. 26.4o/oo		Exp. sal. 25.1°/oo	
May 13, 1957		May 16, 1957		May 29, 1957	
Slope = -.141		Slope = -.203		Slope = -.161	

Table VI. Effect of starvation on the standard metabolic rate of winter Platichthys stellatus. X = body weight in grams, Y = rate of oxygen consumption in mgm. O_2/gm./hr.

Days Fasted

2				11	
X	Y	X	Y	X	Y
2.5	.103	49.7	.0355	4.0	.0455
3.9	.085	54.0	.0435	5.6	.0415
4.8	.0655	57.0	.0465	5.7	.0435
5.2	.058	70.1	.035	8.5	.042
5.5	.076	80.7	.0435	9.7	.041
6.5	.055	81.0	.047	12.0	.0375
7.8	.051	91.0	.046	13.0	.0375
8.1	.063	100	.0345	48	.0315
9.2	.0525	134	.0345	51	.0365
9.8	.079	138	.0435	71	.0308
10.3	.047	157	.044	79	.034
12.1	.0505	240	.0435	80	.0435
13.4	.051	264	.0355	130	.0355
21.8	.063	284	.0385		

Exp. temp. $10 \pm .1^{\circ}C.$
Exp. sal. $24.7\text{-}26.0^{\circ}/oo$
December 15, 1956 and
December 20, 1956
(2 groups)

Exp. temp. $10 \pm .1^{\circ}C.$
Exp. sal. $25.7^{\circ}/oo$
December 24, 1956

The adjusted mean rates of oxygen consumption for each regression of summer and winter fish were calculated from the covariance analyses by the formula (adj $\bar{Y}i = \bar{Y}i - b\ (\bar{X}i - \bar{X})$). The drop in standard metabolic rate of the spring fish is essentially logarithmic and the adjusted means of regression may be fitted with a straight line when plotted on a logarithmic grid (Fig. 10b). The means of the winter rates are included for comparison, though it can only be assumed that the drop is logarithmic because of the absence of a third time period in this group.

Since no fed controls were followed along with the fasted flounder, it is uncertain whether the systematic fall in respiratory rate is due entirely to starvation. A small part of the chage might be caused by thermal acclimation during this period if discrepancies existed between the true environmental temperature and the laboratory temperature. As pointed out before, it was never possible, because of unknown vertical movements of the fish population sampled, and tidal changes with corresponding temperature variation at the surface, to measure either the true mean environmental temperature or the range of temperatures in the environment. However, discrepancies between true acclimation temperature and laboratory temperature must be small at most (less than $1.5^{\circ}C.$). It seems likely that essentially all of the progressive fall in metabolic rate can be attributed to caloric starvation.

Regardless of whether the drop in metabolic rate is due entirely to starvation or partially to starvation and partially to some other influence such as thermal acclimation, the drop will henceforth be referred to as a starvation effect. Data collected for the effect of salinity on metabolic rate was corrected for starvation by referring any two group comparisons to the same day of fasting. For instance, if the metabolic rates of fresh water adapted flounder fasted four days are being compared to a control group of sea water flounder fasted three days, the individual metabolic rates of the four-day fasted group are divided by a fraction graphically derived from an enlarged graph of Figure 10b to bring the metabolic rates to a three-day fasted level.

The question arises as to whether or not the depletion of stored energy reserves during fasting with the systematic fall in metabolic rate in any way interferes with normal osmotic exchange in the flounder. In total starvation (abrupt and complete food deprivation) the body must fall back on stored food reserves to provide energy for metabolic activity. At the outset, carbohydrates (mostly liver glycogen) are consumed to provide calories, but the quantity is small; within a short time the body must rely entirely on fat and tissue proteins for energy. The result is a progressive drop in weight and, in poikilotherms, a fall also in respiratory intensity. However, the diminution of metabolic rate and weight do not fall in the same manner. Smith (1935 a,b) has followed the respiration of fasted African lungfish, Protopterus arthiopicus for periods in excess of 600 days. Whereas weight fell slowly at first and in a nearly linear fashion, oxygen consumption fell exponentially to 50% of the initial value within the first 7 days of the fast. His data depict an approximately straight line when plotted on double logarithmic paper. By following mixed and non-protein R.Q. throughout, Smith found that the amount of carbohydrate burned was small and largely consumed within a few days. For the remainder of the fast until near death over a year and a half later, the lungfish subsisted on stored fat reserves. The amount of tissue protein consumed was very small until late in the fast when a gross combustion of this component occurred concomitant with a sharp terminal increase in oxygen consumption just before death.

Starvation appears to have little effect on the work capacity of fish, at least until the terminal stages of inanition. Barrett (unpublished, cited by Fry, 1957) demonstrated that fasted Salmo gairdnerii can perform muscular work as well as fed controls and consume essentially as much oxygen in active metabolism experiments as did the controls. Many fish cease feeding entirely during spawning migration with no apparent decrease in swimming ability. However, careful measurements have shown a progressive though small decrement in the swimming ability of

fasting Pacific salmon as they migrate up the Columbia River (Paulik and DeLacy, 1958).

Thus, there seems little doubt that sufficient energy exists for osmoregulation, for even the capacity to perform muscular work suffers little or no impairment. In this investigation the salinity effect experiments were carried out during the first few days of the fast. During this time, carbohydrates and fats supply nearly all of the energy needed. It is not until late in starvation, whem combustion of tissue proteins commences, that it is possible to tax severely homeostatic mechanisms. For this reason, it seems a reasonably safe assumption that the few days of starvation imposed on the experimental flatfish had a completely insignificant effect on normal osmotic exchange.

4. Effect of Salinity on the Standard Metabolic Rate of Starry Flounder

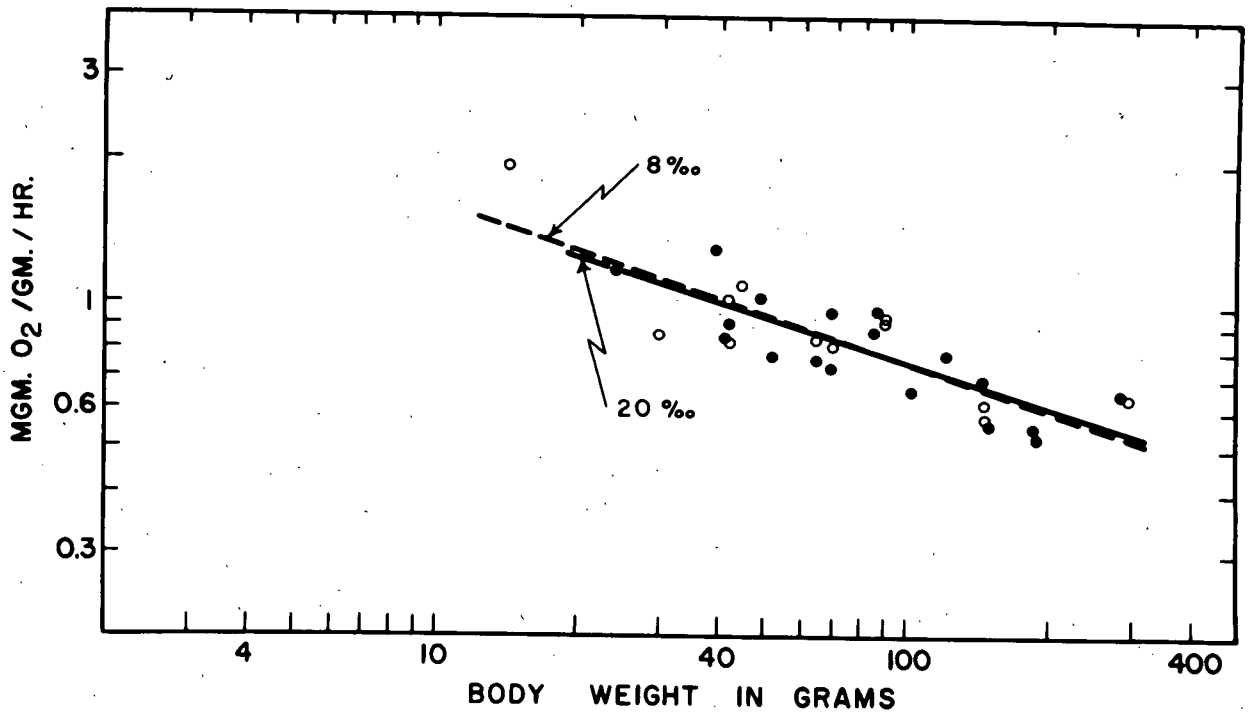
These experiments were carried out during the summer and winter of 1956 and spring of 1957. Three experimental salinities were chosen: the "normal" environmental salinity of about 25°/oo, concentrated sea water of 45°/oo, and fresh water.

Pilot experiments revealed that moderate changes in the osmotic gradient were inadequate to demonstrate differences in standard metabolism. An example is shown in Figure 11a and the data are summarized in Table VII. In this experiment the standard metabolism of 18 flounder was measured in an experimental salinity of 20°/oo (Δ 1.076°C.), the prevailing environmental salinity on that date. Two days later, oxygen consumption measurements were repeated on 12 fish of the same group in a salinity of 8.0°/oo (Δ 0.43°C.) after the standard 20 hour adaptation period in the respirometers. The results, corrected for the systematic fall in metabolic rate due to starvation, were compared by covariance analysis and found to be non-significant, (Appendix-table IV). Essentially no change occurred either in the adjusted treatment means or in the slopes of the fitted lines.

Flounder introduced into fresh water show a significant drop in meta-

Figure 11a. Comparisons of standard metabolic rate of Platichthys stellatus in 20 °/oo (closed circles) and 8 °/oo (open circles) sea water. Respiratory rates fitted by the method of least squares. Experimental temperature 15.2 ±.2°C. The regression lines are not significantly different. Data of Table VII.

Figure 11b. Comparison of standard metabolic rate of Platichthys stellatus in 20 °/00 sea water (closed circles) and after 20 hours in fresh water (open circles). Respiratory rates fitted by the method of least squares. The regression lines are significantly different at the 5% level of probability. Experimental temperature 15.2 ±.2°C. Data of Table VII.


3
1
0.6
0.3
MGM. O_2 /GM. / HR.
8‰
20 ‰
4
10
40
100
400
BODY WEIGHT IN GRAMS

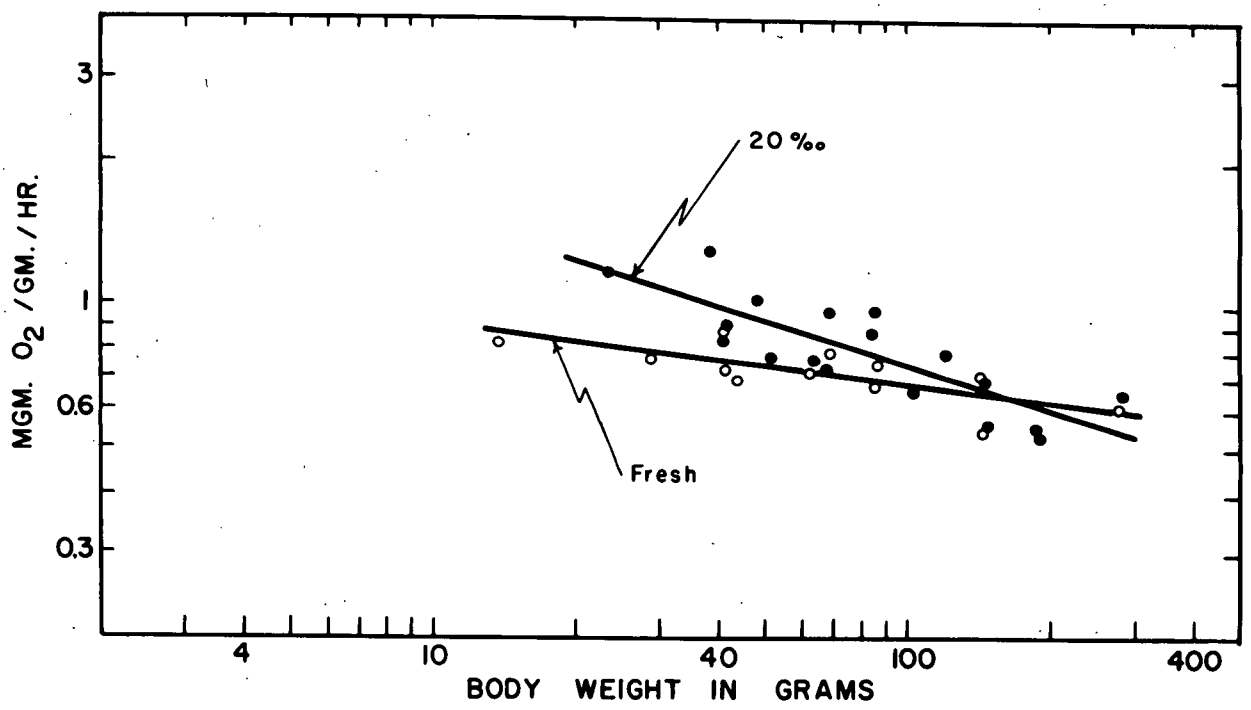

3
1
0.6
0.3
MGM. O_2 /GM. / HR.
20 ‰
Fresh
4
10
40
100
400
BODY WEIGHT IN GRAMS

Table VII. The standard metabolic rate of Platichthys stellatus in 20°/oo sea water and fresh water. 1956 summer studies. X = body weight in grams, Y = rate of oxygen consumption in mgm. O_2/gm./hr. (average of 2-3 determinations).

20°/oo sea water

X	Y
23.4	.117
38.4	.13
40.3	.0845
41.1	.091
48.2	.103
51.6	.078
62.9	.0765
67.2	.074
68.4	.097
84.1	.097
84.4	.0885
101.9	.066
120.1	.079
142.2	.07
148.2	.056
187.6	.055
188.6	.053
283.7	.065

Exp. temp. 15.0±.2°C.
Exp. sal. 20.0°/oo
Days unfed, 3
June 29, 1956
Slope - .311

8°/oo sea water

X	Y	Corrected Y
13.9	.18	.194
28.9	.079	.0854
41.0	.094	.1017
41.4	.0765	.0827
43.3	.1005	.1087
63.3	.079	.0854
68.0	.075	.0811
86.6	.087	.0941
87.7	.084	.0909
143.8	.057	.0616
146.1	.053	.0573
282.5	.059	.0638

Exp. temp. 15.3±.2°C.
Exp. sal. 8.0°/oo
Days unfed, 5
July 1, 1956
Slope - .331
Starvation correction
to day = Y/.924

Table VII (Continued)

Fresh water, 20 hr. adaptation

X	Y	Corrected Y
13.9	.075	.0834
28.9	.071	.0789
41.0	.08	.0889
41.4	.066	.0734
43.3	.063	.0700
63.3	.065	.0723
68.0	.072	.0800
86.6	.068	.0756
87.7	.061	.0678
143.8	.064	.0711
146.1	.049	.0545
282.5	.055	.0611

Exp. temp. 15.1±.1°C.
Exp. sal. 0
Days unfed, 6
July 2, 1956
Slope = -.126
Starvation correction
to day 3 = Y/.899

Fresh water, 4 day adaptation

X	Y	Corrected Y
11.7	.074	.0774
19.0	.074	.0774
19.5	.068	.0712
24.1	.069	.0722
47.8	.055	.057
59.7	.0615	.0643
80.4	,059	.0617
81.7	.0495	.0518
84.5	.055	.0575
90.0	.049	.0513
94.7	.058	.0607
145.5	.057	.0596
171.2	.0525	.0549
196.2	.0525	.0549

Exp. temp. 15.0±.2°C.
Exp. sal. 0
Days unfed, 6
July 9, 1956
Slope = -.135
Starvation correction
to day 3 = Y/.955

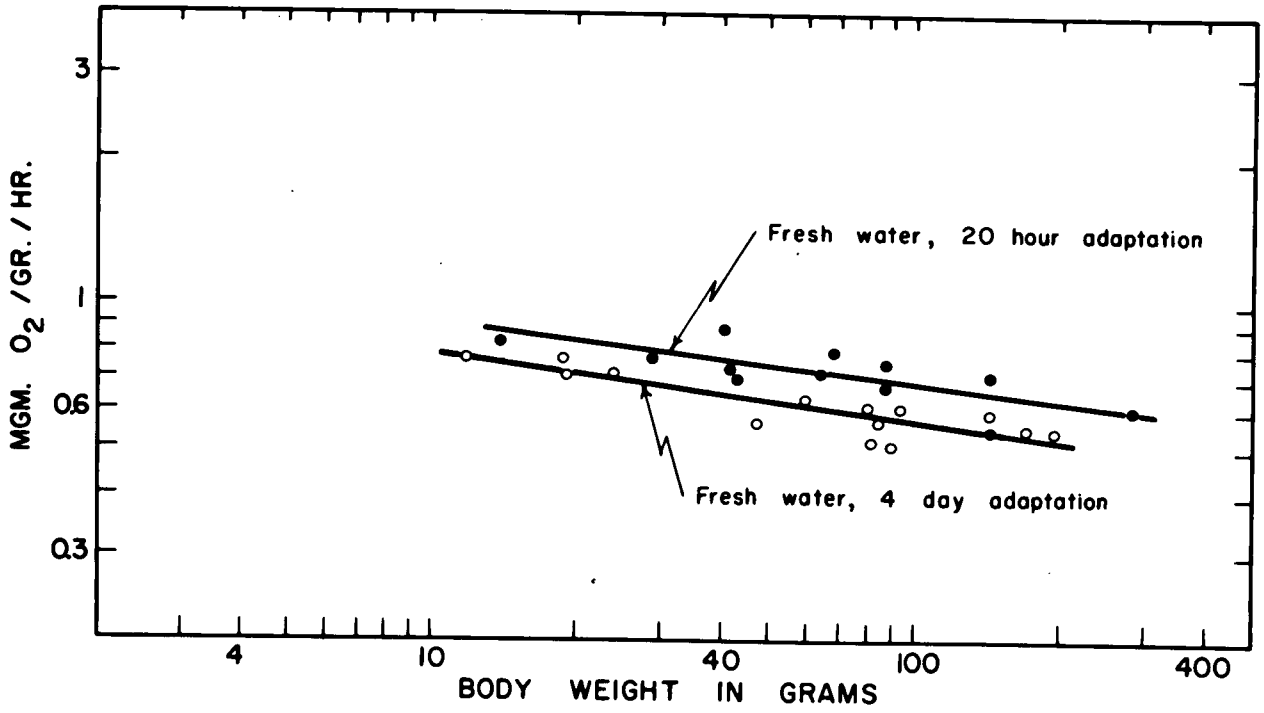
bolic rate. The decrease in oxygen consumption appears to be gradual and becomes increasingly marked after the fish have adapted to fresh water. In Figures 11b and 12a the oxygen consumption of two groups of flounder in fresh water are compared with a control group in 20°/oo sea water and with each other. In the first group (Fig. 11b), measurements were made 20 hours after abrupt transfer of the flounder to fresh water. Although the data are significantly different at the 5% probability level ($F_{.01} - 7.68 > F = 7.5 > F_{.05} = 4.21$), the difference is primarily a prominent change in slope due to a drop in total metabolism of the small fish only. In Figure 12a, these fresh water flounder are again compared with another group previously adapted to fresh water for 4 days before metabolic rates were determined. In this interval a highly significant drop in metabolic rate has occurred ($F = 29.1 > F_{.01} = 7.88$). The drop (16% difference between the treatment means) is represented in both small and large flounder, for there was essentially no change in slope. These experiments were carried out in June and July, 1956. The data are summarized in Table VII and the statistical tests are given in Appendix-table IV. All data were corrected for the effect of starvation so that treatment comparisons are of adjusted data representing flounder of the same nutritional state.

In December, 1956, an experiment first was conducted to measure the effect of high salinity on oxygen consumption of flounder. In Figure 12b, Table VIII, metabolic rates of 13 flounder determined after 20 hours in 49°/oo sea water show a marked increase above the control group of 28 flounder in 25°/oo sea water. The differences are highly significant at the 1% probability level ($F = 55.1 > F_{.005} = 8.83$; Appendix-table V). Thus, the metabolic demands for osmoregulation of flounder in sea water increases with increasing salt content of the water.

Another series of experiments was performed in May, 1957, which in effect serve to replicate the studies already described. These data are given

Figure 12a. Effect of adaptation time in fresh water on the standard metabolic rate of Platichthys stellatus. 20 hour adaptation rates are represented as closed circles and 4 day adaptation as open circles. Respiratory rates are fitted by the method of least squares. The regression lines are significantly different at the 1% level of probability. Data of Table VII.

Figure 12B. Comparison of standard metabolic rate of Platichthys stellatus in 25 °/oo sea water (closed circles) and in 49 °/oo sea water (open circles). Respiratory rates are fitted by the method of least squares. The regression lines are significant at the 1% level of probability. Experimental temperature 9.9 ±.2°C. Data of Table VIII.


3
1
0.6
0.3
MGM. O_2 /GR. / HR.
Fresh water, 20 hour adaptation
Fresh water, 4 day adaptation
4
10
40
100
400
BODY WEIGHT IN GRAMS

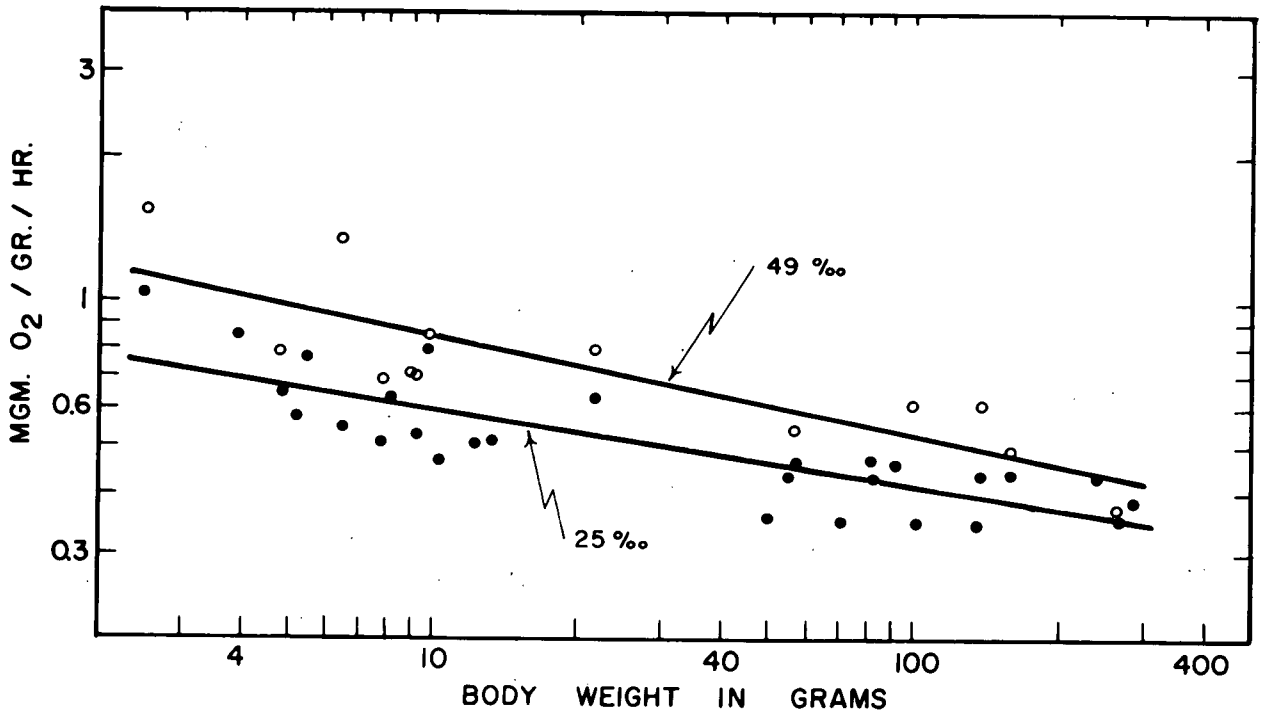

3
1
0.6
0.3
MGM. O_2 / GR. / HR.
49 ‰
25 ‰
4
10
40
100
400
BODY WEIGHT IN GRAMS

Table VIII. Standard metabolic rate of Platichthys stellatus in 25°/oo and 49°/oo sea water. 1956 winter studies. X = body weight in grams, Y = rate of oxygen consumption in mgm. O_2/gm./hr. (average of 2-3 determinations).

25°/oo sea water

X	Y	X	Y
2.5	.103	49.7	.0356
3.9	.085	54	.0435
4.8	.0655	57	.0465
5.2	.058	70.1	.035
5.5	.076	80.7	.0435
6.5	.055	81	.047
7.8	.051	91	.046
8.1	.063	100	.0347
9.2	.0527	134	.0346
9.8	.079	138	.0433
10.3	.047	157	.044
12.1	.0505	240	.0435
13.4	.051	264	.0354
21.8	.0628	284	.0384

Exp. temp. = 10.0±.1°C.
Exp. sal. 24.7 - 26.0°/oo
December 15, 1956 and
December 20, 1956 (two groups)
Days unfed = 2
Slope = -0.16

49°/oo sea water

X	Y	Corrected Y
2.5	.142	.1525
4.8	.073	.0784
6.5	.125	.1342
7.8	.064	.0687
9.0	.0659	.0707
9.2	.0645	.0692
9.8	.0795	.0853
21.8	.0741	.0795
57.0	.0505	.0542
100	.057	.061
138	.0566	.0607
157	.0455	.0488
264	.0346	.0371

Exp. temp. = 9.8±.1°C.
Exp. sal. = 49.2°/oo
Days unfed = 3
December 21, 1956
Slope = -0.203
Starvation correction
to day 2 = Y/.931

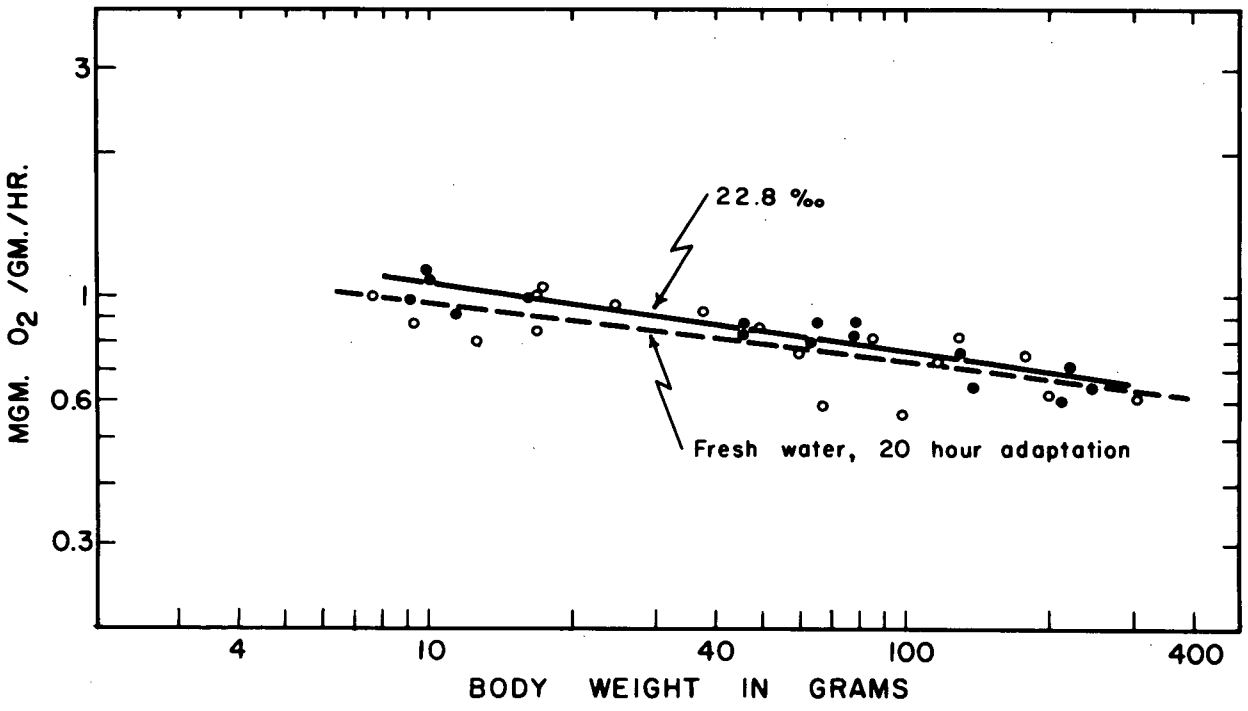
in Table IX. Standard metabolism studies were conducted on flounder at two fresh water adaption periods, first at 20 hours (Fig. 13a) and again at 5 days (Fig. 13b) after introduction into fresh water from sea water. Similar measurements were carried out on flounder 20 hours after introduction into a salinity of 43°/oo from 25°/oo (Fig. 14). Since the observations were not carried out simultaneously, but over a period of several days, each experiment represents groups of fish each in distinct nutritional states. As before, the data were adjusted by reference to the exponential slope representing the mean rate of fall of metabolic rate due to starvation (see Figure 10b). The starvation effect thus removed, the data can be compared statistically and graphically for changes due to salinity.

Figure 13a shows that a small decrease in standard metabolism has occurred in flounder 20 hours in fresh water as compared with sea water controls of the same nutritional state (8% decrease in the mean rate of oxygen consumption). The decrease is significant at the 10% but not at the 5% probability level ($F_{.05} = 4.17 > F = 3.04 > F_{.10} = 2.88$). After 5 days in fresh water, (Fig. 13b), the standard metabolism of fresh and salt water flounder in the same nutritional state have diverged to a 10.5% difference in mean rates. This drop in metabolic rate (difference in the regression means) is highly significant (Appendix-table VI). These results are in accordance with those of the summer, 1956, series which showed that flounder adapted 4 days to fresh water had relatively lower mean metabolic rates than those in fresh water for only 20 hours.

The percentage decrease in metabolic rate of flounder transferred to fresh water is dependent on body size and is greater in small than in large animals. This observation is based on the decrease in slope of the regression line through the oxygen consumptions of fresh water flounder as compared with their salt water controls. The slope of the 20 hour adapted fresh water

Figure 13a. Comparison of standard metabolic rate of Platichthys stellatus in 22.8 °/oo sea water (closed circles) and in fresh water, 20 hour adaptation (open circles). Respiratory rates fitted by the method of least squares. The regression lines are significantly different at the 10% but not at the 5% probability level. Experimental temperature 15.0 ±.1°C. Data of Table IX.

Figure 13b. Comparison of standard metabolic rate of Platichthys stellatus in 25 °/oo sea water (closed circles) and in fresh water, 5 day adaptation (open circles). Respiratory rates are fitted by the method of least squares. The regression lines are significantly different at the 1% probability level. Experimental temperature 14.8 ±.1°C. Data of Table IX.


3
1
0.6
0.3
MGM. O_2 /GM./HR.
22.8 ‰
Fresh water, 20 hour adaptation
4
10
40
100
400
BODY WEIGHT IN GRAMS

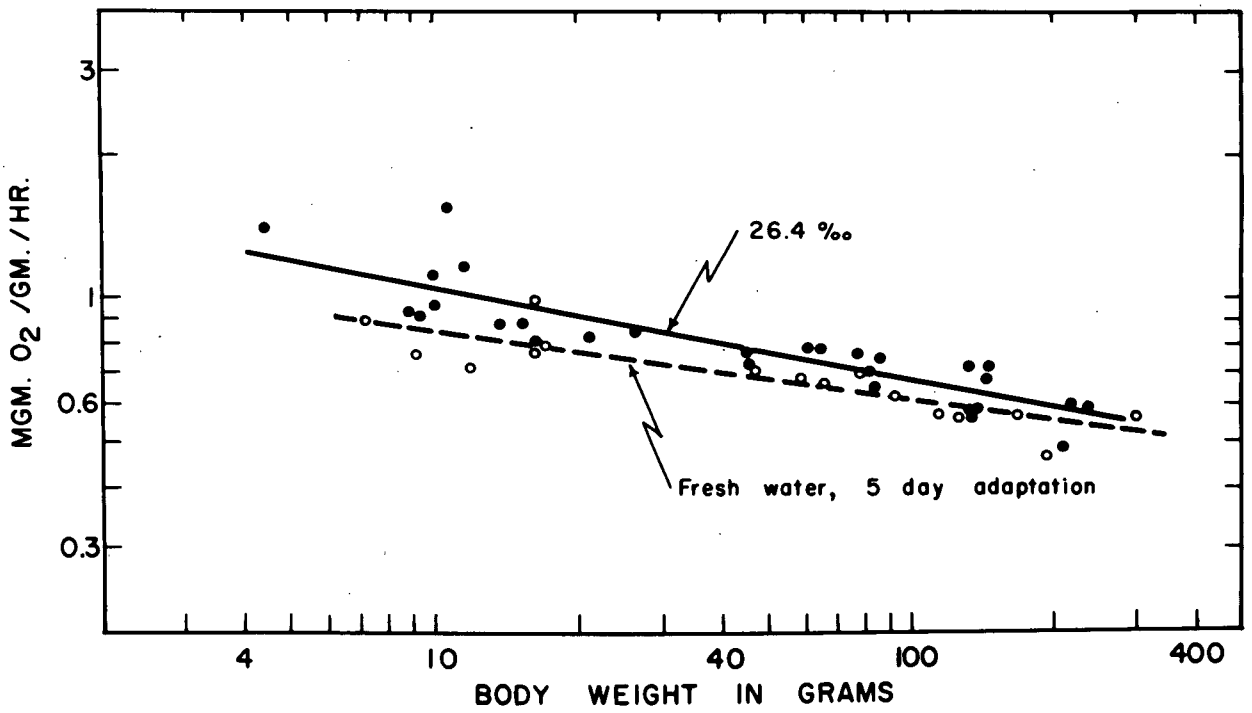

3
1
0.6
0.3
MGM. O_2 /GM./HR.
26.4 ‰
Fresh water, 5 day adaptation
4
10
40
100
400
BODY WEIGHT IN GRAMS

Table IX. Standard metabolic rate of Platichthys stellatus in fresh water, normal sea water and concentrated sea water. 1957 spring studies. X = body weight in grams, Y = rate of oxygen consumption in mgm. O_2/gm./hr. (average of 2-3 determinations).

22.8°/oo sea water		Fresh water, 20 hour adaptation			43.2°/oo sea water, 22 hour adaptation		
				Corrected			Corrected
X	Y	X	Y	Y	X	Y	Y
9.0	.099	7.6	.0975	.1008	5.3	.134	.1308
9.9	.114	9.2	.0855	.0884	7.1	.123	.1201
10.1	.109	12.6	.0775	.0801	8.4	.143	.1396
11.3	.092	16.6	.097	.1003	11.0	.118	.1152
16.0	.10	16.8	.082	.0848	18.1	.105	.1025
45.6	.084	17.1	.102	.1054	18.4	.098	.0957
45.6	.0894	24.6	.0925	.0956	23.0	.122	.1191
62.4	.081	36.9	.090	.093	31.0	.100	.0976
65.6	.0893	48.0	.0835	.0863	48.4	.077	.0751
78.0	.0845	59.5	.0736	.0761	63.2	.096	.0937
79.0	.088	67.7	.0565	.0584	68.0	.090	.0878
132.3	.0766	83.5	.0785	.0811	94.3	.070	.0683
139.2	.0645	97.4	.0544	.0562	148.1	.105	.1025
212.1	.0618	116.0	.071	.0734	204.4	.067	.0654
223.6	.071	128.0	.0795	.0822	229.5	.0755	.0736
245.6	.065	175.8	.073	.0754	286.4	.0775	.0756
		197.7	.0603	.0623			
		306.5	.059	.061			
Exp. temp. = 15.0°C.		Exp. temp. = 15.0±.1°C.			Exp. temp. = 14.0±.1°C.		
Exp. sal. = 22.8°/oo		Exp. sal. = 0			Exp. sal. = 43.2°/oo		
Days unfed = 4		Days unfed = 5			Days unfed = 6		
May 13, 1957		May 14, 1957			May 22, 1957		
Slope = -0.141		Slope = -0.124			Slope = -0.151		
		Starvation correction to day 4 = Y/.967			Starvation correction to day 7 = Y/1.024		

Table IX. (Continued).

25-26.4°/oo sea water

X	Y	X	Y
4.4	.14	64.5	.079
8.8	.093	75.0	.065
9.2	.091	76.6	.0775
9.8	.111	81.5	.071
10.0	.097	85.9	.076
10.6	.156	131.7	.058
11.7	.116	131.7	.072
13.6	.088	131.8	.075
15.3	.089	136.7	.0596
16.5	.081	145.7	.073
21.3	.0825	146.1	.068
26.6	.085	205.5	.049
44.8	.071	215.7	.06
45.7	.077	236.9	.0595
61.1	.079		

Exp. temp. = 14.8±.1°C.
Exp. sal. = 25.0 and 26.4°/oo
Days unfed = 7
May 16, 1957 and May 23, 1957 (two groups)
Slope = -0.192

Fresh water, 5 day adaptation

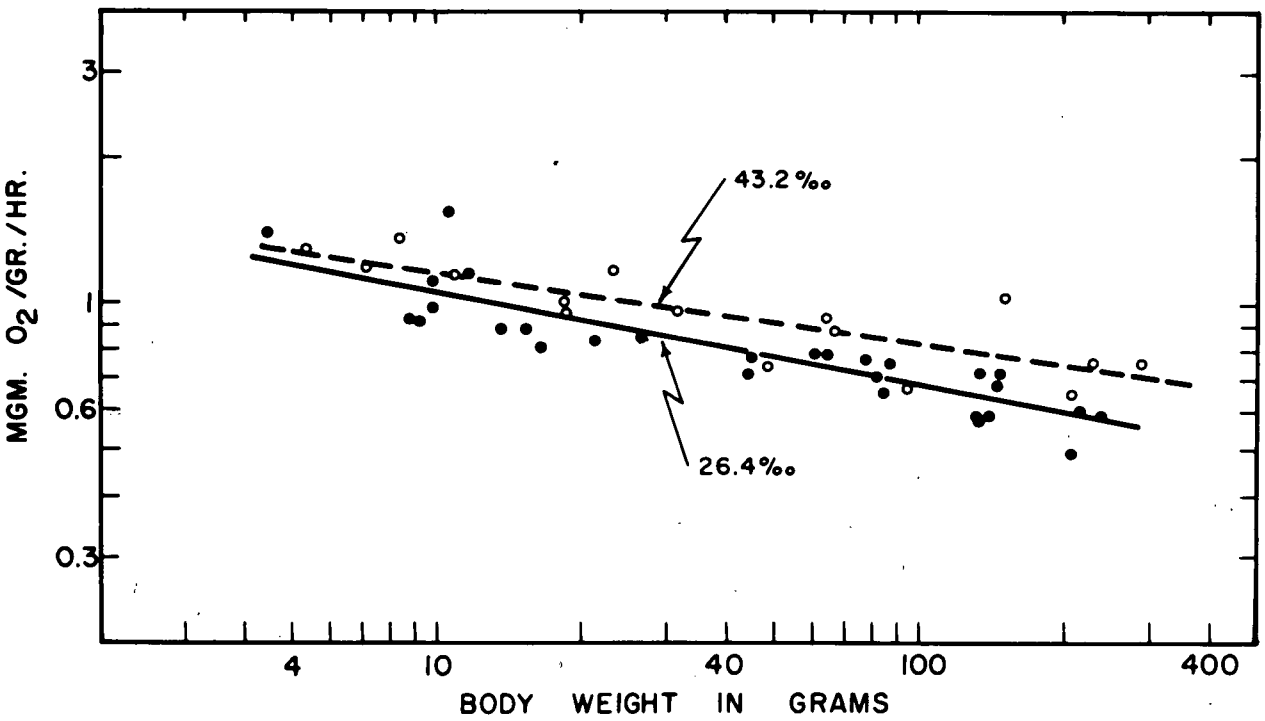
X	Y	Corrected Y
7.1	.086	.0898
9.1	.0734	.0766
11.9	.069	.072
16.1	.075	.0783
16.2	.096	.1002
16.5	.079	.0825
47.0	.068	.071
57.9	.066	.0689
66.1	.0645	.0673
79.4	.069	.072
92.5	.0604	.063
114.9	.055	.0574
127.5	.054	.0564
166.4	.0543	.0567
194.3	.045	.047
299.5	.0542	.0566

Exp. temp. = 14.7±.1°C.
Exp. sal. = 0
Days unfed = 9
May 18, 1957
Slope = -0.142
Starvation correction
to day 7 = Y/.958

flounder is -.124 and -.140 for the control group. For the 5 day fresh water adapted flounder the slope is -.142 as compared to -.192 of the salt water controls. The decrease is small but consistent and is in agreement with the 1956 series which showed even greater decreases in slope.

The mean standard metabolic rate of flounder in 43°/oo (Fig. 14, Table IX) is 15.1% above the mean of the control group. The difference is highly significant, with a variance ratio of 17.3 (Appendix-table VI). These results agree with the winter, 1956, experiment (Fig. 12b) though the mean increase in metabolic rate is not as great (15.1% in the summer experiment as opposed to a 25% increase in the winter). The slopes of regression, though different from the controls in both cases, are inconsistent in the direction of change and preclude any generalization as to a possible interaction between body size and salinity effect so far as the high salinity is concerned. In the spring 1957 experiment, the slope is less than that of the control groups (-.151 and -.192 respectively) and may indicate a relatively greater energy expenditure for osmotic regulation in the large than in the small animals. In the winter experiment, however, the slope became steeper in the high salt concentration (-.203 at 49°/oo and -.16 at 25°/oo) but the results represent only 13 fish with considerable variability in the oxygen consumption rates. Thus, these results, while unquestionably demonstrating a higher expenditure of energy in the high salt concentration for all fish, are inadequate with respect to showing whether the energy demands in the high salinity are relatively greater for small than large flounder, or vice versa. In fresh water, on the other hand, energy demands for osmoregulation are significantly less. The results as well show a consistent decrease in slope in the weight-specific oxygen consumption of flounder in fresh water, implying that osmoregulatory demands for energy are relatively less for small than large flounder in fresh water as compared with the energy expenditure of small and large salt water flounder.

Figure 14. Comparison of standard metabolic rate of Platichthys stellatus in 25 °/oo sea water (closed circles) and in 43.2 °/oo sea water (open circles). Respiratory rates fitted by the method of least squares. The regression lines are significantly different at the 1% probability level. Experimental temperature 14.0 - 14.8°C. Data of Table IX.


3
1
0.6
0.3
MGM. O_2 /GR./HR.
43.2‰
26.4‰
4
10
40
100
400
BODY WEIGHT IN GRAMS

5. The Effect of Salinity on the Standard Metabolic Rate of Lemon Sole and Speckled Sand Dab

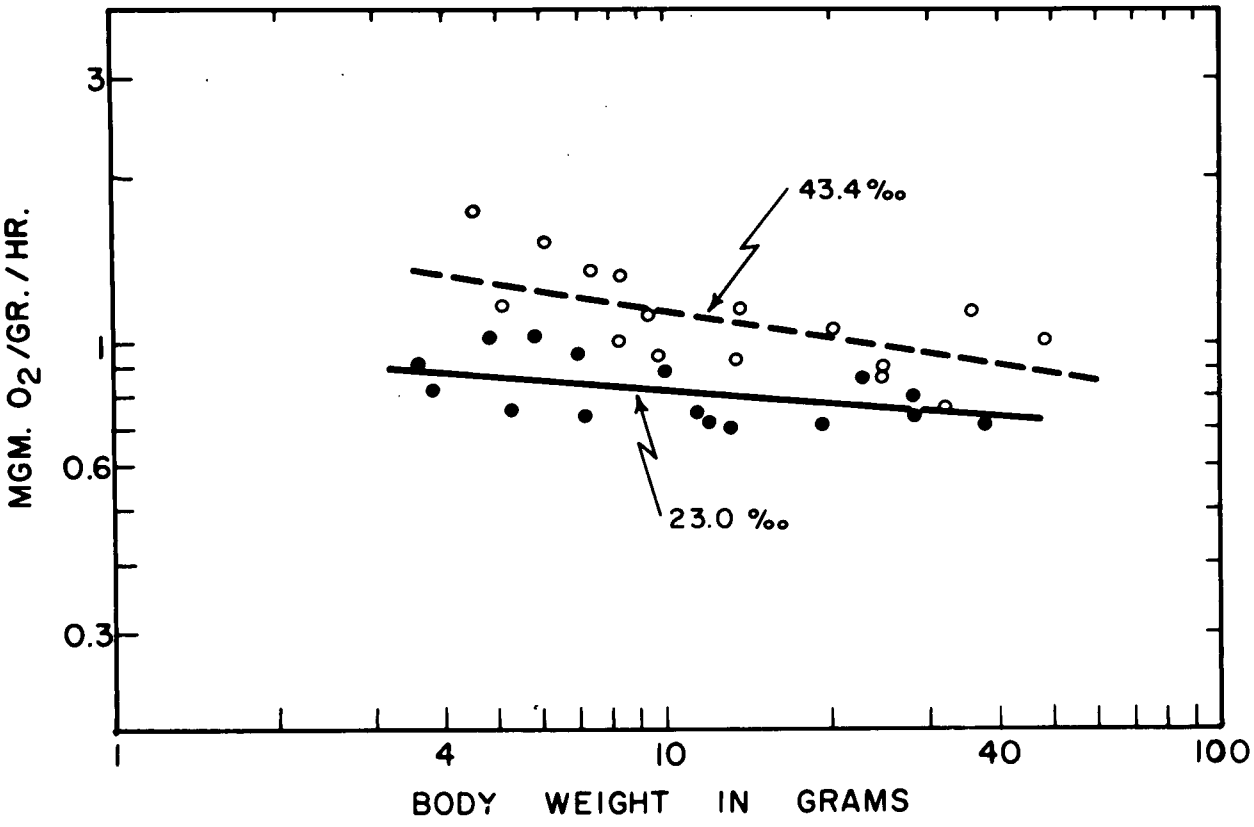
Because an adequate supply of lemon sole (Parophrys vetulus)and speckled sand dab (Citharichthys stigmaeus) was unpredictable at best and actually unavailable most of the time, it was not possible to replicate completely with these species the salinity effect experiments carried out with flounder. Only scattered experiments were conducted when an adequate size range of either species was collected. The two experiments here presented, one on the sole, the other, the sand dab, are of some value as a comparison of the influence of salinity on the respiration of these stenohaline forms with the euryhaline starry flounder.

Figure 15a, Table X shows that an increase in the ambient salt concentration is accompanied by a marked increase in total metabolic rate of the speckled sand dab. The experiment was carried out after a 3 day adaptation period in concentrated sea water of 43 p.p.t. (Δ 2.275°C.) and is compared with a control group of the same nutritional state (fasted 3–4 days) in 23°/oo sea water (Δ 1.24°C.). The difference in the means of regression are highly significant with a variance ratio of 44.6 ($F_{.01}$ = 7.56, Appendix-table VII). There is also an increase in slope in the high salinity (b = -.203 as compared with -.095 for the controls). This suggests that the actual amount of energy expended for osmoregulation as a percentage of the total metabolic rate is influenced by body size, with a greater increase in energy expenditure for osmotic work in small than in large sand dab. This interpretation must be accepted with caution because it was not possible to replicate the experiment. It should be noted that the respiratory response of sand dab exposed to increased salinity is the same as that of starry flounder in increased salinity - an overall increase in total metabolic rate.

As pointed out in the introduction, the measurement of metabolic changes attending the transfer of a marine fish into a sea water dilution below

Figure 15a. Comparison of standard metabolic rate of Citharichthys stigmaeus in 23 °/oo sea water (closed circles) and in 43.4 °/oo sea water (open circles). Respiratory rates are fitted by the method of least squares. The regression lines are significantly different at the 1% level of probability. Experimental temperature 14.9 ±.2°C. Data of Table X.

Figure 15b. Comparison of standard metabolic rate of Parophrys vetulus in 24.3 °/oo sea water (closed circles) and in 5.8 °/oo (open circles). No significant change in metabolic rate occurred although individual variability was greatly increased in the group in the low salinity. Experimental temperature 15.0 ±.2°C. Data of Table XI.


3
1
0.6
0.3
MGM. O2 /GR. / HR.
43.4‰
23.0 ‰
1
4
10
40
100
BODY WEIGHT IN GRAMS

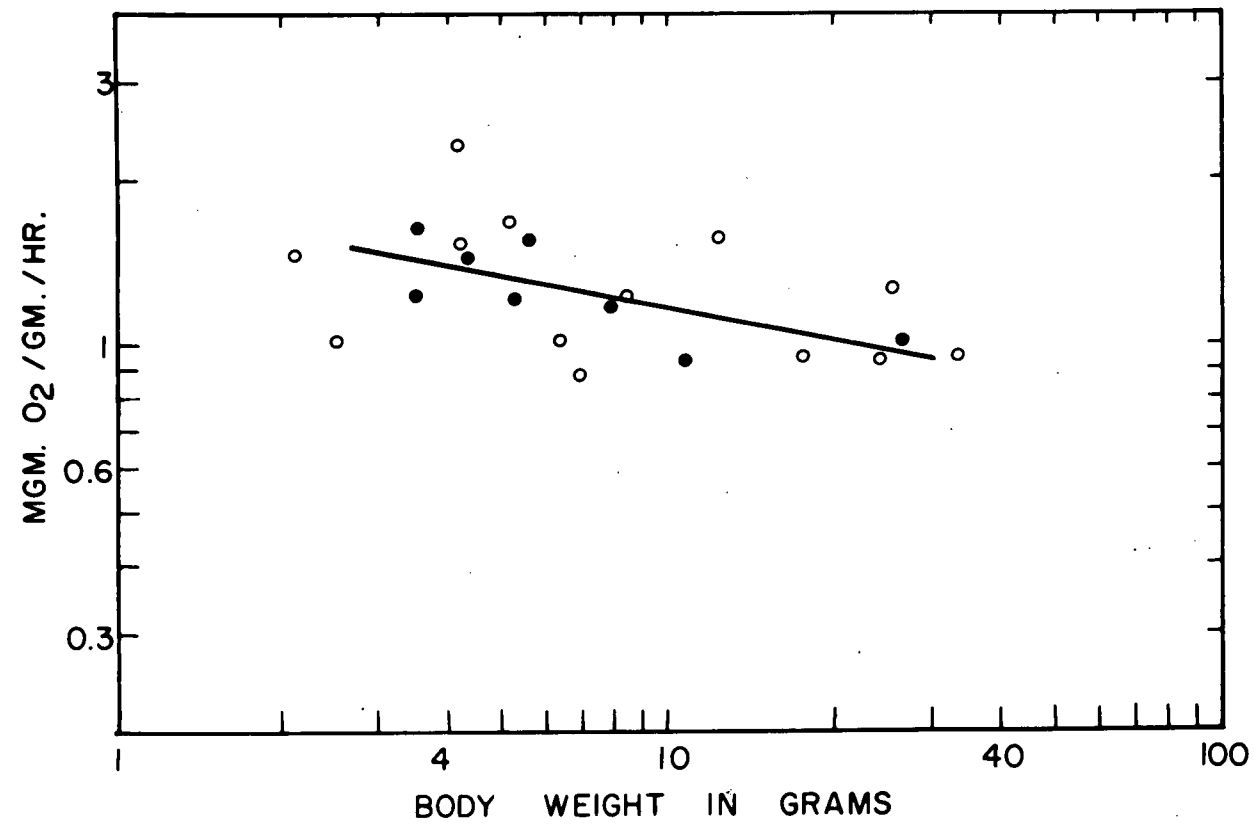

3
1
0.6
0.3
MGM. O2 /GM. / HR.
1
4
10
40
100
BODY WEIGHT IN GRAMS

Table X. Standard metabolic rate of Citharichthys stigmaeus in 24.4°/oo and 43.4°/oo sea water. X = body weight in grams, Y = rate of oxygen consumption in mgm. O_2/gm./hr. (average of 2-3 determinations).

24.4°/oo		43.4°/oo	
X	Y	X	Y
3.6	.092	4.55	.176
3.8	.082	5.05	.118
4.9	.102	6.0	.154
5.2	.0748	7.25	.137
5.8	.103	8.1	.101
7.0	.0955	8.3	.134
7.1	.074	9.3	.113
10.3	.082	9.8	.094
11.6	.0746	13.5	.093
12.3	.071	13.9	.116
13.1	.070	20.3	.108
19.1	.071	24.4	.0905
22.9	.086	24.5	.088
28.1	.080	31.4	.076
28.3	.074	35.2	.115
38.0	.0706	47.9	.101
Exp. temp. = 14.8±.1°C.		Exp. temp. = 15.0±.1°C.	
Exp. sal. = 24.4°/oo		Exp. sal. = 43.4°/oo	
July 5 and 6, 1957		July 15 and 16, 1957	
Days unfed = 3 and 4		Days unfed = 4 and 5	
Slope = -0.095		Slope = -0.203	

Table XI. Standard metabolic rate of Parophrys vetulus in 24.35°/oo and 5.8°/oo sea water. X = body weight in grams, Y = rate of oxygen consumption in mgm. O_2/gm./hr. (average of 2-3 determinations).

24.35°/oo		5.8°/oo	
X	Y	X	Y
3.5	.165	2.1	.145
3.5	.123	2.5	.101
4.3	.145	4.1	.23
5.3	.123	4.2	.152
5.7	.155	4.2	.149
8.0	.117	5.2	.169
10.8	.094	6.5	.101
26.9	.101	6.9	.088
		8.4	.121
		12.3	.157
		17.8	.095
		24.1	.094
		25.3	.126
		33.4	.095

Exp. temp. = 14.9±.1°C.
Exp. sal. = 24.35°/oo
Days unfed = 3
July 21, 1957
Slope = -0.153

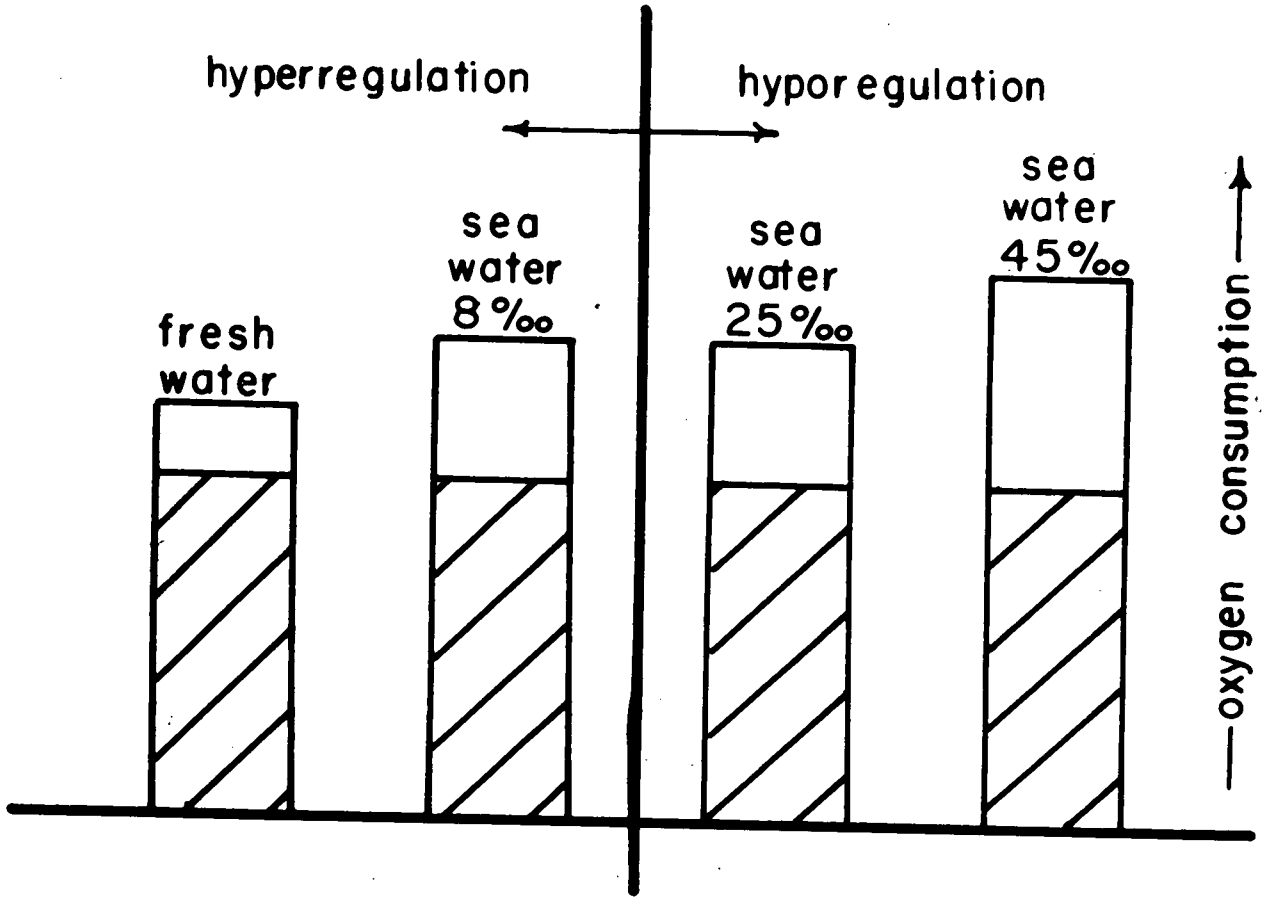
Exp. temp. = 15.1±.1°C.
Exp. sal. = 5.8°/oo, adapted 3 days
Days unfed = 4 and 5
July 22 and 23, 1957
Slope = -0.15

the incipient lethal salinity level has questionable physiological significance. Nevertheless, the procedure offers an interesting comparison between the metabolic responses of euryhaline and comparatively stenohaline marine fishes introduced into low salinities. Such an experiment was performed and is illustrated in Figure 15b (Table XI). The standard metabolic rates of a group of lemon sole were determined three days after transfer from normal sea water to a salinity of 5.8°/oo (0.31). The results show that essentially no change has occurred in the mean metabolic rate of these sole as compared with the controls, but that there has been a large increase in individual variability. Lemon sole generally survive less than a week in 6°/oo sea water. In this experiment some mortality occurred at the time of the oxygen consumption determinations; the attendant variability in metabolic rate reflects the moribund condition of the sole.

C. COMMENT

It has been shown that the starry flounder is both a hypotonic and hypertonic regulator, possessing compensating mechanisms for internal osmotic regulation in the face of abrupt and drastic alteration or reversal of osmolarity of the external environment. These alterations were characterized by significant changes in metabolic activity of the flounder which are assumed equivalent to changed energy needs for osmotic work. An understanding of osmotic energetics necessitates consideration of all processes utilized to maintain internal equilibrium regardless of external osmotic vicissitudes. Starry flounder consumed less oxygen in fresh water than in normal sea water and still greater energy demands were made in supernormal salinities. These relationships are shown diagrammatically in Figure 16. Any proposed explanation for these events must of necessity explain the apparent increasing

Figure 16. Diagrammatic representation of relative energy demands of starry flounder for osmotic regulation in hypotonic and hypertonic media. Oxygen consumption of "basal" cellular metabolism other than processes connected with osmoregulation are represented by the lower cross-hatched portion of the bars. This portion is assumed to be unaffected by changes in slinity. The upper clear portion of the bars represents variable oxygen demands for osmoregulation depending on the environmental salinity.


hyperregulation
hyporegulation
fresh water
sea water 8‰
sea water 25‰
sea water 45‰
oxygen consumption

osmotic work with increasing salt content of the environment. The true explanation lies in relative metabolic demands for the transportation of electrolytes and water in the principal organs of exchange, the gills, the kidney and the gut. The importance of the integument in limiting permeability to salts and water cannot of course be overemphasized, but permeability of fish skin is a passive attribute of scales and mucus, rather than an active energy consuming process. A "differential" permeability may or may not exist but until such a phenomena is demonstrated it must be assumed that the diffusion of water through the body surface is dependent only on the concentration and direction of the gradient on either side.

Therefore, the relative osmotic work loads of the individual organs of exchange must be considered, but here one is hampered by our meager knowledge of the interrelationships of these organs, the relative osmotic demands made on them and their efficiency in doing the job. While no direct measurements have been made of the oxygen consumption of the perfused teleost kidney or gill doing osmotic work, the recent disclosures of Ussing (1958) are of great value in predicting energy demands of these organs for ion transport. In his study of active ion transport by the frog skin, Ussing found that it costs as much oxygen to transport equivalent amounts of sodium across a low concentration gradient as across a high concentration gradient. Oxygen consumption is dependent on the <u>amount</u> of sodium transported regardless of whether the movement is uphill or not. Ussing demonstrated further that 16 to 20 sodium ions were transported for each molecule of oxygen consumed. Thus, with the frog skin at least, and very probably in all biological membranes, an inseparable quantitative relationship exists for energy demands of the ion transport mechanism.

This new quantitative thermodynamic concept of active transport provides a convenient basis to a possible explanation for the observed lower

oxygen consumption of flounder in fresh water than in sea water. Since the salinities used in these experiments represent tonicities of essentially equal though opposite osmotic gradient (fresh water of Δ 0 < body fluid of Δ 0.65 < sea water of Δ 1.35) it can be assumed that the hydrating and dehydrating effects of fresh and salt water respectively are nearly equal in their disturbing influence on water balance. The difference is found in salt metabolism. In order to maintain osmotic equilibrium the fresh water fish excretes osmotic water in a copious urine of low specific gravity. Small amounts of salt are continually lost in the urine and are regained from ingested food and by ion absorbing cells in the gills and mucous membranes (Krogh, 1937; Copeland, 1948, 1950; Wikgren, 1953). The marine fish, however, has adopted a much more circuitous method for regaining lost osmotic water, that of drinking sea water and excreting the salts via the gills. This method entails more "handling" of salts by the organs of exchange. Salt must be transported across the gastrointestinal mucosa to the blood, carried to the gills and again secreted across membranes to the external milieu. The process is wasteful of precious body water since the ingested hypertonic sea water must be diluted to isotonicity in the gut (Smith, 1930, and unreported observations in this laboratory on the starry flounder). While univalent ions (sodium, chloride and small quantities of potassium) are absorbed from the gut and excreted by the gills, most divalent ions are retained and concentrated in the intestinal residue (Smith, 1953). Some divalents do enter the circulation and are excreted by the kidney, accompanied by an obligatory water loss since the flounder is unable to concentrate urine even to isotonicity (Figs. 1a and 1b). Again active transport is necessary, either to resorb univalent ions from the glomerular filtrate or, what is probably more important in view of the relative unimportance of the latter (Forster, 1953), to secrete selectively divalents into the urine by the tubules from the renal portal circulation. All of this

complex ion movement is necessitated by the inability of marine fish to separate specifically water molecules from sea water for direct absorption. It appears, in fact, that no animal cell can pump water into the cell against an osmotic gradient (though water can be pumped out of a cell against an osmotic gradient after it has passively diffused in). Therefore, the distillation of sea water by marine teleosts obligates much electrolyte transfer with a concomitant expenditure of energy. In contrast to this, fresh water fishes have only the relatively simple task of replacing the small amount of salt lost in the urine. Water is free for the fresh water teleost and this, of course, causes its major osmotic problem - disposing of water without losing salt. It is generally assumed that since fresh water fish with but one known exception possess glomerular kidneys, the dilute and copious urine is formed from the glomerular filtrate, an ultrafiltrate of the arterial blood supply to the kidney. However, no measurements of either renal blood flows or glomerular filtration rates have been carried out on fresh water fish. In view of the importance of the renal portal supply to the teleost kidney, it is altogether possible that a greater or lesser portion of the urine represents tubular secretion of water or solutes from this venous supply. The one exception to the apparent universality of glomerular kidneys of fresh water teleosts, the aglomerular pipefish, Microphis boaja, is significant, for it means that tubular secretion alone is adequate for life in fresh water.

Numerous measurements of oxygen consumption by the mammalian kidney have been reported and are summarized by Smith (1951). In general, oxygen consumption correlates directly with renal blood flow so that as the latter is decreased so follows the oxygen consumption. Water diuresis has no influence on renal oxygen consumption. These findings would appear to have new meaning in the light of Ussing's disclosure of the precise relationship between the amount of sodium transported and the energy (in terms of oxygen consumption)

needed for the process. Increased renal blood flow means an increased filtration rate and greater ion reabsorptive activity by the tubule, hence the greater oxygen consumption. Water diuresis would have no effect since the work of filtration is supplied by the heart through the arterial pressure. As long as the filtration rate remains the same, no quantitative alteration in ion reabsorption would occur in spite of the greater urine flow.

Since the rate of glomerular filtration almost certainly increases when the euryhaline flounder moves from salt to fresh water (though studies of this interesting change in renal function are wholly lacking), a concomitant increase in tubular reabsorptive activity for the formation of hypotonic urine would be expected. Whether this process of the fresh water flounder kidney consumes more energy than the secretion of divalent ions by the kidney of the marine flounder is an open question. Our present lack of knowledge on kidney function in fishes is a prominent obstacle in the way of a clear understanding of teleost osmoregulation. The work of Forster and Berglund (Forster, 1953; Forster and Berglund, 1956; Berglund and Forster, 1958) has contributed much to an understanding of renal function in marine fishes, but in fresh water fishes the picture remains obscure.

The results of the standard metabolism experiments show an apparent contradiction, viz: the oxygen consumption of flounder is less in fresh water than it is in 8°/oo, a salinity approaching isotonicity (Fig. 11a). Presumably energy demands should be minimal in the absence of an osmotic gradient to tax energy consuming homeostatic mechanisms. However, there is no a priori reason to believe that all osmotic mechanisms cease when a euryhaline fish enters isotonic brackish water. It is conceivable that marine starry flounder continue to drink water after entering low salinities, although the process would no longer seem to be efficacious. As long as salt is transported through membranes regardless of the osmotic gradient, energy demands for osmoregulation

continue. Again, the lack of experimental evidence precludes further speculation as to the reason for the observed relatively high oxygen consumption in brackish water.

In supernormal salinities (45-50°/oo) the metabolic rate of starry flounder and speckled sand dab was found to increase considerably above the rate in normal sea water (25°/oo). As the external salt concentration increases, the osmotic loss of body water through mucous membranes also increases. To restore fluid balance, more sea water must be ingested, the salt separated and excreted. Since the ingested water is more saline, a much greater quantity of salt must be transported for each gram of water absorbed by the gut. Not only are extrarenal water losses augmented, but it becomes increasingly difficult to replace these losses. In addition, the flounder must excrete more divalent ions via the kidneys and because the urine remains hypotonic even in concentrated sea water (Fig. 1b), the obligatory renal water losses are greater. Though no measurements have been made, we would theoretically expect an <u>increase</u> in urine flow with a rise in the external osmotic gradient. A certain relief from this mushrooming problem in water conservation is obtained by allowing the concentration of the body fluids to rise somewhat (Fig. 1b). If careful metabolism studies were made over a range of salinities, it would probably be found that the oxygen consumption increases exponentially rather than linearly with an increasing osmotic gradient. The net result of high salinities is that a greater portion of the animal's basal energy requirements must be devoted to maintaining homoiosmoticity. Since fishes are strictly limited in the amount of oxygen available for cellular respiration both by gill limitations and because of the low oxygen tensions in the aquatic environment, any increase in basal metabolic demand is distinctly detrimental to a species, since it decreases the respiratory reserve for activity. Maximal oxygen consumption (active metabolism) is never far above

standard metabolic rates, even in active species such as trout or salmon (Job, 1955; Fry, 1957). In a high salinity, the "scope for activity" (Fry, 1947) or the difference between active and standard metabolic rates, is considerably decreased as a result of augmented energy demands for osmotic work and also because oxygen solubility in water decreases with increasing salt content. Decreases in activity of salmon smolt (Salmo salar) moving into salt water from fresh water have been observed (Huntsman and Hoar, 1939). In this laboratory, Houston (1958) has measured significant decreases in locomotor activity of chum salmon (Oncorhynchus keta) moved from fresh to sea water. Although it is uncertain whether decreased activity in sea water is the result of lowered "scope for activity" or is due to a direct inhibitory action on muscle fibers of changes in electrolyte composition of the blood, it is evident that high salinities reduce the physiological reserve of fish for other environmental demands.

V

THE EFFECT OF ENVIRONMENTAL SALINITY ON THYROID ACTIVITY AND RADIOIODIDE METABOLISM OF THE STARRY FLOUNDER

The frequently implicated role of the teleost thyroid in water and electrolyte metabolism has remained obscure. The problem has been approached both with respect to measured alterations in thyroid activity effected by salinity changes and to induced changes in salinity tolerance by administering thyroid preparations. The literature on this subject has been reviewed by Fontaine (1953, 1956), Hoar (1951, 1957), Smith (1956) and Pickford and Atz (1957). If a generalization can be ventured from the existing evidence, some of which is contradictory, it appears that thyroid activity decreases with increasing salinity of the external environment. Freshwater species transferred to dilute sea water frequently show a transitory thyroid inactivation more noticeable in those species with particularly active glands in fresh water (Olivereau, 1950, 1954). Conversely, marine species maintained in hypotonic salinities usually develop hyperactive thyroids. Hoar (1952) found that landlocked smelt (Osmerus mordax) and alewives (Pomolobus pseudoharengus) always had more active thyroids than individuals collected from coastal estuaries. The landlocked alewives had extremely hyperplastic glands and experienced heavy mortality during the reproductive season when demands on thyroid hormone are evidently increased. Leloup (1948) and Olivereau (1948, 1954) noted a transitory decrease in thyroid activity of two marine species, Muraena helena and Labrus bergylta, subjected to dilute sea water followed by a gradual return to normal activity in the lowered salinity. Transfer of the marine killifish, Fundulus majalis, from sea water of 25.5°/oo to dilute sea water of 5.1°/oo caused a small (25%) increase in thyroid uptake of I^{131} (Gorbman and Berg, 1955), while the same treatment with the brackish water Fundulus heteroclitus

resulted in a very much greater (150%) increase in the peak I^{131} uptake of fish in the lower salinity.

The most convenient explanation for these findings is that fresh water fish have a greater physiological demand for thyroid hormone in a direct or adjunctive osmoregulatory role. A more likely explanation, however, lies in the low iodine levels of fresh water as compared with sea water. Since Marine and Lenhart in 1910 demonstrated that thyroid hyperplasia in brook trout, Salvelinus fontinalis could be completely abolished by adding small amounts of iodine to the water, several authors have shown that thyroid hyperactivity in fresh water species is frequently a goitrogenic reaction to the low iodine content of fresh water. Both the uptake of radioiodine (Berg and Gorbman, 1953, 1954; La Roche, 1953) and histological criteria (Hamre and Nichols, 1926; La Roche, 1950; Hoar, 1952; Robertson and Chaney, 1953; Schlumberger, 1955) have been used to demonstrate hyperplasia and to show that the condition was immediately alleviated by adding even minute amounts of iodine to the water. Lack of iodine prevents normal production of thyroid hormone, resulting in a lowered titer of hormone in the blood. With the normal inhibiting action of thyroid hormone removed, more thyroid-stimulating hormone from the anterior pituitary is produced with the result that the thyroid follicles become distended with an incomplete form of thyroglobulin or "colloid".

Not all experimental findings can be explained on the basis of the iodine content of the water. Among the most interesting experiments are those of Koch and Heuts (1943) and Heuts (1943) who found that feeding thyroid to the euryhaline stickleback, Pygosteus markedly decreased the resistance of this species to sea water. The treatment appeared to have a direct effect on mineral metabolism; chlorides accumulated in the blood and the animals eventually died. Fresh water sticklebacks were unaffected by thyroid feeding. Similarly, Baggerman (1957) found that thyroxine administration induced a

fresh water preference and thiourea a salt water preference in the three-spined stickleback, Gasterosteus.

Fontaine and Baraduc (1954) found that while a single feeding of iodinated casein decreased the salinity resistance of Salmo salar, prolonged treatment with thyroxine or iodinated casein induced certain morphological changes (pseudo-smoltification) and a concomitant increase in salinity resistance. Thus the length of treatment appears to be an important factor in inducing changes in salinity tolerance. Baggerman (1957) also demonstrated a reversal in the induced salinity preference of Gasterosteus. While thyroxine induced fresh water preference during the first few days of treatment, a return to salt water preference occurred on the seventh day. Similarly, thiourea treatment caused sticklebacks to pass transitionally through a period of fresh water preference, then return to salt water preference (Exps. 38A and 38B, Fig. 20, of Baggerman's paper). However, in other experiments, the reversal in induced salinity preference did not occur during the period of treatment. These results and those of Fontaine and Baraduc (1954) may possibly explain the results of Koch and Heuts (1942) who reported decreased salinity tolerance of sticklebacks after a single feeding of thyroxine.

The recent work of Burden (1956) and Smith (1956) strongly suggests that at least for the species studied, killifish and trout, an osmoregulatory role of the thyroid, if present, is collateral or subsidiary to some other endocrine influence. Smith's work with Salmo trutta showed that while thyroxine in high dosages promoted salinity resistance, growth hormone was far more effective in this respect. Burden's work indicates the involvement of an unknown pituitary factor in osmoregulation of Fundulus. Thyroxine therapy of hypophysectomized fish had no effect on the inability of these animals to survive in fresh water. Gorbman (in discussion following paper by Smith, 1956) found that thiourea treatment had no effect on the normal

euryhalinity of Fundulus heteroclitus. Treated fish with inhibited thyroid glands resisted transfer to either fresh or salt water as well as controls.

It is doubtful that either the administration of thyroid material or the inhibition of thyroid function with chemical inhibitors are effective or justifiable ways to demonstrate an osmoregulator role of the thyroid gland. One criticism is that any therapy may have unknown side-effects that could alter the normal physiology of the species. Until fundamental research, presently lacking, reveals the effects of thyroid inhibitors, such as thiourea or thiouracil, on all aspects of the fishes' metabolism, they should be used with caution. Similarly the feeding or injection of thyroid substance may have far-reaching effects not immediately apparent. For one thing, thyroid administration inhibits secretion of TSH resulting in a marked hypoactivity of the animal's own thyroid gland. Another argument against such treatment is that it is difficult to know when or whether the treatment has been effective. As already discussed, the apparent effect induced by treatment may actually reverse if the treatment is continued long enough.

The teleost thyroid is perhaps the most labile of the endocrine glands. Just about all of the environmental factors known to influence the total metabolism of the animal, such as season, body size, sex, sexual maturity, reproductive cycles and temperature have been shown influential on thyroid activity. These factors have been discussed in several reviews such as those by Hoar (1951, 1957), Lynn and Wachowski (1951) and Pickford and Atz, (1957) and will not be reiterated here. To evaluate thyroid function with respect to one specific effect, such as the role of the thyroid in osmotic regulation, demands a careful appraisal of many other environmental influences. Factors such as temperature, body size and iodine content of the water can be experimentally controlled. Seasonal and reproductive cycles cannot be controlled but can be accounted for in the experimental design. Therefore it is impossible to divorce the study of an osmoregulatory role of the thyroid gland

from other direct or collateral effects.

Several criteria for evaluating thyroid activity are available, but only two, histological and radiological, have been used extensively by fish physiologists. Of the radiological methods, the simple uptake of radioiodine by the thyroid gland has become a research tool of considerable importance. Tests usually attempt to quantitate the amount of radioiodine trapped by the thyroid either by measuring with counting instruments the proportion of the dose accumulated by the excised gland or by some quantitative interpretation of autoradiographs prepared from the gland. At least two distinct mechanisms are responsible for the uptake of iodine by the thyroid gland. The first is the "iodide trap" and the second is the utilization of the accumulated inorganic iodide to synthesize organic thyroid hormone. Radioiodine tests of thyroid activity of fishes most commonly have been of the form of uptake curves, where the percentage fraction of a single tracer dose of I^{131} accumulated by the thyroid is plotted against the time after administration of the dose. "In vivo" measurements of I^{131} accumulation using external counting arrangements have not yet been developed for use with fishes, so that in practice, a large number of fish must be given a standard dose simultaneously, sacrificed at intervals of time thereafter, the thyroids removed and their radioactivity counted. The percentage thyroid uptakes of individual fish are plotted against time of sacrifice to form a composite uptake curve. Injections are usually made intraperitoneally. These curves appear in most cases to be a reliable estimate of the true thyroid activity (the rate of secretion of thyroid hormone) under many experimental conditions. Two very important factors, however, limit the diagnostic accuracy of radioiodine tracer studies in fishes. One is the amount of elemental iodide in the ambient water already mentioned. The other equally important factor is the disappearance rate of the tracer dose from the blood. It is clear that collection of radioiodine by the thyroid gland is

totally dependent on the amount of isotope delivered to the gland by the blood stream. Other things equal, less I^{131} will accumulate in the thyroid when the injected dose is rapidly removed from the body than when removal is slower. If the excretion rates of radioiodine differ under different experimental conditions, it may not be justifiable to use thyroid I^{131} uptake curves as a comparison of thyroid activity between experimental treatments. Salinity variations particularly would be expected to produce changes in iodide behavior in the body fluids because of the marked quantitative and directional alterations in electrolyte and water movements across the organs of exchange - the kidney, gills, oral membranes and alimentary canal. Temperature variations should also influence iodide excretion rates, since metabolic rate and concomitantly the rate of electrolyte exchange of poikilothermic animals such as fish are directly dependent on the environmental temperature. For these reasons it must be concluded that thyroid I^{131} uptake in itself is an unreliable parameter for evaluating thyroid activity if either salinity or temperature are altered as experimental treatments.

To evaluate thyroid activity of flounder in fresh and salt water, another parameter, that of thyroid clearance has been chosen. This appears to satisfy the requirements for a test that is essentially uninfluenced by variations in the disappearance rate of radioactive iodide from the blood. Thyroidal radioiodide clearance, expressed as the volume of blood cleared of its radioiodide per minute, is actually a modification of the standard renal clearance formulae:

$$I^{131}\ \text{Clearance, thyroid} = \frac{\text{thyroid } I^{131} \text{ uptake during t minutes}}{\text{mean blood conc. of } {}_{1}131 \text{ during t minutes.}}$$

Since the dose of radioiodide is disappearing exponentially from the blood and because following the early phase of iodine uptake I^{131} -labeled hormonal iodine begins to appear in the blood, only the first few hours after injection are suitable for clearance calculations.

In the present study, thyroid activity of the starry flounder as influenced by salinity has been studied with radioiodine, paying particular attention to the behavior of the tracer dose in the body fluids. With the exception of some preliminary work by Chavin (1956b) essentially nothing is known of iodine metabolism in fishes. A large portion of the research was devoted, therefore, to radioiodine movements - its excretion, distribution in the body and behavior in the blood - in both fresh and salt water flounder. In comparing thyroid activity in fresh and salt water, flounder were pre-adapted to fresh water containing an added amount of iodide equivalent to the iodine content of sea water at the experimental salinity used. As with the foregoing sections of this thesis on body fluid concentration and metabolic rate, attention has been given to the effect of size on thyroid activity.

A. METHODS: DETERMINATION OF IODIDE MOVEMENT AND THYROID ACTIVITY WITH RADIOIODINE

1. Injections

Radioiodine in the form of carrier-free sodium iodide was diluted with saline or distilled water for injection. For any one experimental series, the dosage of I^{131} injected into each fish was the same, usually 5 microcuries per fish. When serial sampling of blood was carried out, larger doses (20-30 microcuries per fish) were administered to ensure adequate radioactivity of the small samples for counting. The volume of injected fluid containing the I^{131} was always 0.05 ml. per fish. Injections were made intraperitoneally with a 0.25 ml. tuberculin syringe and 27 gauge needle. To prevent leakage of the injected from the blind side by passing the needle at an acute angle through the ventral musculature and into the posterior portion of the coelom. The muscle thus acted as a seal against fluid leakage after withdrawal of the needle.

Five aliquots of each dose were reserved as standards and given appropriate dilutions with slightly alkaline water for counting with the samples.

2. Collection, Treatment and Counting of Samples.

a. Thyroid

As with most teleosts, the thyroid of the flounder is a diffuse gland with thyroid follicles scattered widely about the ventral aortae. By sectioning this area of the lower jaw of flounder previously injected with I^{131} and counting the radioactivity of individual small portions, it was determined that the thyroid always lay anterior to the third branchial arch with some extension of follicles a short distance laterally along the gill bars. Hence, in collecting thyroid samples as much non-thyroidal tissue as possible was trimmed from the lower jaw area without infringing on the region where thyroidal tissue was known to lie.

During the course of the study three separate counting instruments were used. Initially a Geiger-Müeller counter was employed. Later, a scintillation well counter and finally an end-probe scintillation counter became available. Consequently, treatment of the thyroid sample in preparation for counting depended on the counting instrument used. For beta-ray counting with the end-window Geiger-Müeller counter, the thyroid samples were placed in 15 ml. test tubes, calibrated at 10 ml. and wet-ashed with 5 ml. of 2N NaOH. Following ashing and cooling, the solution was diluted with water to the 10 ml. mark, stirred and allowed to settle. A one ml. aliquot of the ash solution was transferred to a stainless-steel planchet 25 mm. in diameter and with a 7 mm. edge. The samples were allowed to evaporate to near-dryness at room temperature, then dried thoroughly under an infrared lamp. Standard samples were prepared by pipetting into planchets 1 ml. aliquots representing 1/100 of the dose. Using a predetermined count scaling unit with the thin mica-end-window Geiger counter, two measurements were made of each sample and standard to 3000 counts. Glassware was

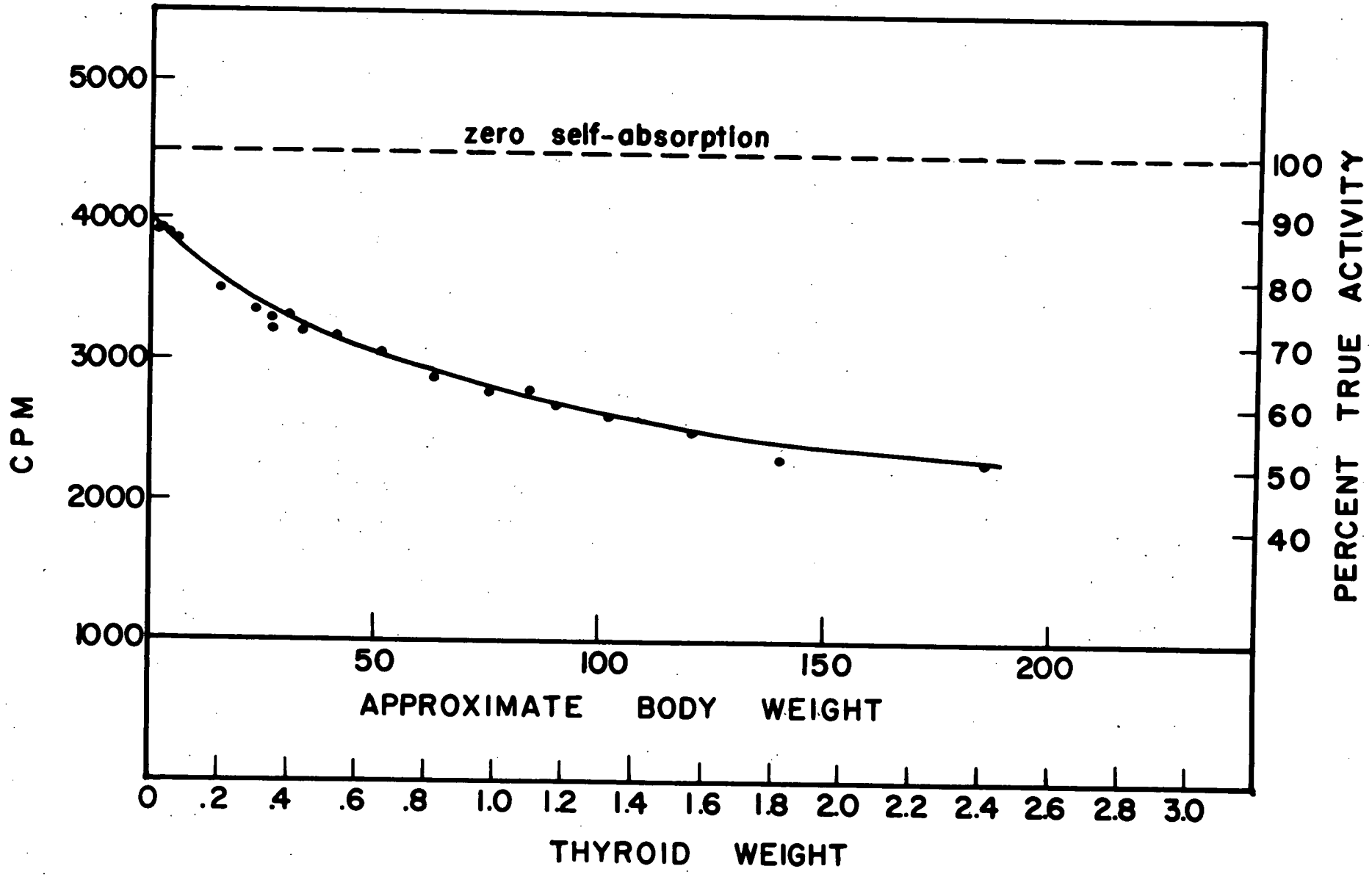
cleaned thoroughly with detergent and potassium iodide carrier between uses and checks showed that there was no transfer of radioactivity between sample solutions.

Since the Geiger-Mueller counter detects mostly beta radiation, "self-absorption" of these particles by the residue in the ashed sample is an important factor which must be accounted for. The effect is particularly important when sample mass varies as is the case when evaluating thyroid activity of fish of different sizes. To quantitate the self-absorption effect a set of standards was prepared using different masses of thyroid samples but with constant amount of radioactivity in each sample. These were ashed in sodium hydroxide, aliquots pipetted to steel planchets, dried and counted with the Geiger-Mueller counter and referred to a standard with no tissue or alkali, representing zero self-absorption. The results (Fig. 17) show that the observed counting rate decreases with increasing thyroid mass in a non-linear (but not exponential) manner. Forty to 50 percent of the radiation may be absorbed by the tissue mass of thyroids of large flounder (100-200 grams). Thyroids of small fish show much less self-absorption, but the effect never approaches zero because of the deposit of sodium hydroxide in the planchet which itself absorbs about 10% of the emitted radiation.

In comparing thyroid I^{131} uptake of fish of the same size, the self-absorption effect can for most purposes be ignored. Serious errors will, however, be introduced if the effect is ignored when studying thyroid activity of fish of different sizes. Since the counting rate of I^{131} is essentially independent of sample mass when counting gamma rays with a scintillation counter, this instrument is much to be preferred over the Geiger counter when working with variable sample masses. No correction for self-absorption is necessary with gamma ray counting.

Most of the studies associated with body size effect on thyroid activity were carried out using the well-crystal scintillation counter as the

Figure 17. The effect of self-absorption on the observed counting rate of I^{131} in thyroid samples as measured with an end-window Geiger-Mueller counter.


5000
4000
3000
2000
1000
CPM
zero self-absorption
100
90
80
70
60
50
40
PERCENT TRUE ACTIVITY
50
100
150
200
APPROXIMATE BODY WEIGHT
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
THYROID WEIGHT

counting instrument.[1] Thyroid samples were collected, trimmed and ashed in sodium hydroxide as before. One ml. aliquots of the samples were pipetted into 5 ml. plastic vials ("Clearsite," 1½ dram size) and diluted to 4 ml. with water. Counting was then accomplished with the scintillation well counter (1½" x 2" NaI (Tl) crystal) and a predetermined count scaler.

During the studies on salinity effect on thyroid activity, counts were made with an end-probe scintillation counter,[2] using a 1¾" diameter x 1½" thick NaI (Tl) crystal. Total sample digestion was unnecessary with this counter, provided the samples and standards were similar in mass and geometry. Thyroid samples were placed directly into steel planchets (25 mm. x 7 mm.) with about 1 ml. of hot 2N NaOH and allowed to set overnight in a warm (70°C.) oven. This simple treatment effectively digested the tissue to spread the radioactivity evenly over the planchet. Standards representing 1/100 dose were prepared and allowed to spread evenly over the planchet bottom. Counting was carried out with the crystal 6 cm. distant from the samples.

b. Blood

Blood was collected at the time each flounder was killed by puncturing the dorsal aortae above the coelom and withdrawing the blood with a clean pipette as it welled up from the wound. The blood sample, amounting to about 1% of the body weight, was placed in a steel planchet with Parafilm cover, tared to 4 decimal places, and the cover firmly folded over the planchet top to prevent evaporation from the blood. The sample was then weighed, the Parafilm cover discarded and 1 ml. 2N NaOH added to the blood in the planchet to dissolve

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1. The scintillation well counter was made available by the British Columbia Medical Research Institute through the kindness of Dr. Peter Solvonuk.

2. The end-probe scintillation counter was made available through the generosity of Dr. Harold Copp and Dr. Carl F. Crammer of the University of British Columbia Department of Physiology.

the clot and spread the radioactivity evenly over the planchet bottom. After drying, samples and standards representing 1/100 dose were counted with the end-probe scintillation counter with the crystal 2 to 6 cm. distant depending on the activity of the sample.

Serial sampling of blood was carried out on three flounder (103, 154 and 213 grams) by direct needle puncture of the caudal artery with a ¼cc. syringe and 27 gauge needle. With some practice, the artery could be readily located by introducing the needle into the caudal hypaxial musculature just ventral to the lateral line and passing the needle inward and forward until the artery was entered. A very small sample of blood (0.03 to 0.1 ml.) was withdrawn, transferred to a tared Parafilm-covered planchet, weighed and digested with NaOH as before. Dryed samples and standard were counted with the end-probe scintillation counter.

c. Urine

Urine samples were collected at irregular intervals. After blotting the body of the flounder dry, urine was expressed into a long, thin pipette placed against the urinary papillae by gently pressing the body wall over the urinary bladder. The amount collected varied considerably - between 0.1 and 1% of the body weight. The sample was placed into a tared, Parafilm-covered planchet, sealed, weighed and counted as described for blood samples.

d. Body

To measure the rate of disappearance of radioactivity from the bodies of living flounder, fish were counted individually with the end-probe scintillation counter. At intervals after injection, flounder were removed from the experimental aquarium, placed on a Lucite tray and the activity counted with the scintillation crystal 18 cm. from the fish. The flounder never struggled during this period if covered with a wet paper towel. After counting, the

flounder was immediately returned to the aquarium.

Disappearance rates of I^{131} from the bodies of flounder were usually compiled from single body counts of individual flounder killed at intervals after injection. After collecting blood and urine samples and removing the thyroid gland, the body of each flounder was placed in a plastic petri dish (90 mm. in diameter with 13 mm. sides) and covered. A flounder too large to fit directly was cut into pieces and fitted into the dish, or, in the case of a very large flounder, divided among 2 or 3 dishes. Standards containing the entire dose were prepared either by injecting fish of representative sizes with the standard dose and immediately killing and placing them in petri dishes or by cutting layers of filter paper to represent roughly the shape of the fish, and adding the standard I^{131} dose to the paper in the petri dish. Bodies and standards of similar mass and geometry were then counted in the end-probe scintillation counter with the crystal 18 cm. from the samples.

3. Expression of Results

The simplest expression of the amount of radioactivity in the thyroid gland is counts per minute, corrected for background count. However, this expression of concentration is adequate as a comparative measure only during one specific dosage and counting arrangement. The usual method for expressing the uptake of I^{131} by the thyroid gland, and the method here adopted, is percentage of dose of I^{131} accumulated by the gland per unit of time after injection. This is the most convenient and certainly the most easily interpreted way to present the results. In these experiments, a standard dose was administered to all flounder regardless of body weight. Hence, the mean activity of the body fluids of a 10 gram fish is 10 times that of a 100 gram fish. However, if the true activity of the thyroids of the 10 and the 100 gram fish are the same they will both take up the same <u>percentage</u> of the dose, since the measurement is of the <u>entire</u> gland rather than a similar unit of mass from both

animals.

The actual weight of the trimmed thyroid tissue as a percentage of the body weight varied considerably, but there appeared to be no unconscious tendency toward more thorough trimming in small or in large fish. The average weight of the trimmed tissue was about 1.3% of the total body weight and varied between 0.935% and 1.535% of the body weight. This variation in the amount of non-thyroided tissue could contribute a significant error to the results, because the thyroid tissue is heavily vascularized and I^{131} quickly diffuses throughout the extracellular space. However, the error is probably significant only during the first one or two hours following injection when the activity of blood and interstitial tissue is high and the actual uptake of I^{131} by the follicles is still low. Later the effect becomes insignificant as I^{131} is rapidly removed from the extracellular space (renal and extrarenal excretion and thyroidal uptake) while the level trapped by the follicles steadily increases.

The amount of radioactivity in the body is also conveniently expressed as percentage of dose. The total excretion of I^{131} between the times of injection and sacrifice of the flounder is then the difference between 100% and the percentage of dose remaining in the body.

The expression of blood and urine activity presents a different sort of problem since we are no longer dealing with entire organs or glands. A ml. blood sample from a 10 gr. flounder would contain 10 times the percentage of dose as a blood sample from a 100 gr. flounder when both fish are given the same dose. Thus the body weight must be taken into account. This is done by multiplying the percentage of dose per gram of blood or urine by the body weight in grams. The expression has been called the "biological concentration coefficient" (Comar, 1955). In this study, the concentration coefficient is divided by 100 to give a value more easily compared to thyroid uptake values.

The calculation of "excretory" clearance (representing all routes of

I^{131} excretion) and thyroidal clearance are simple modifications of the standard renal clearance formula, usually expressed as:

$$\frac{\text{concentration of A in the urine x volume of urine}}{\text{concentration of A in the blood}}$$

or:

$$\frac{\text{rate of excretion of A}}{\text{concentration of A in the blood}}$$

Substance A in this case is I^{131} and the formula becomes:

$$I^{131}\ \text{Clearance, excretory} = \frac{\text{rate of } I^{131} \text{ excretion during t minutes}}{\text{mean blood concentration of } I^{131} \text{ during t minutes}}$$

and

$$I^{131}\ \text{Clearance, thyroid} = \frac{\text{rate of thyroid } I^{131} \text{ uptake during t minutes}}{\text{mean blood concentration of } I^{131} \text{ during t minutes}}$$

I^{131} is disappearing exponentially from the blood and its mean concentration may be calculated graphically by plotting I^{131} concentration on a log scale against time on a linear scale, or by the formula:

$$\bar{B} = \frac{B_1 - B_2}{\ln B_1 - \ln B_2}$$

where B is the concentration of I^{131} in percentage of dose (concentration coefficient) in the blood. Thyroidal and renal clearance of I^{131} is discussed by Myant and Pochin (1949); Myant, Pochin and Goldie (1949); Berkson, et.al. (1950); Keating, et.al. (1950), and Berson et.al. (1952).

B. RESULTS

1. Factors Influencing the Excretion of Radioiodide

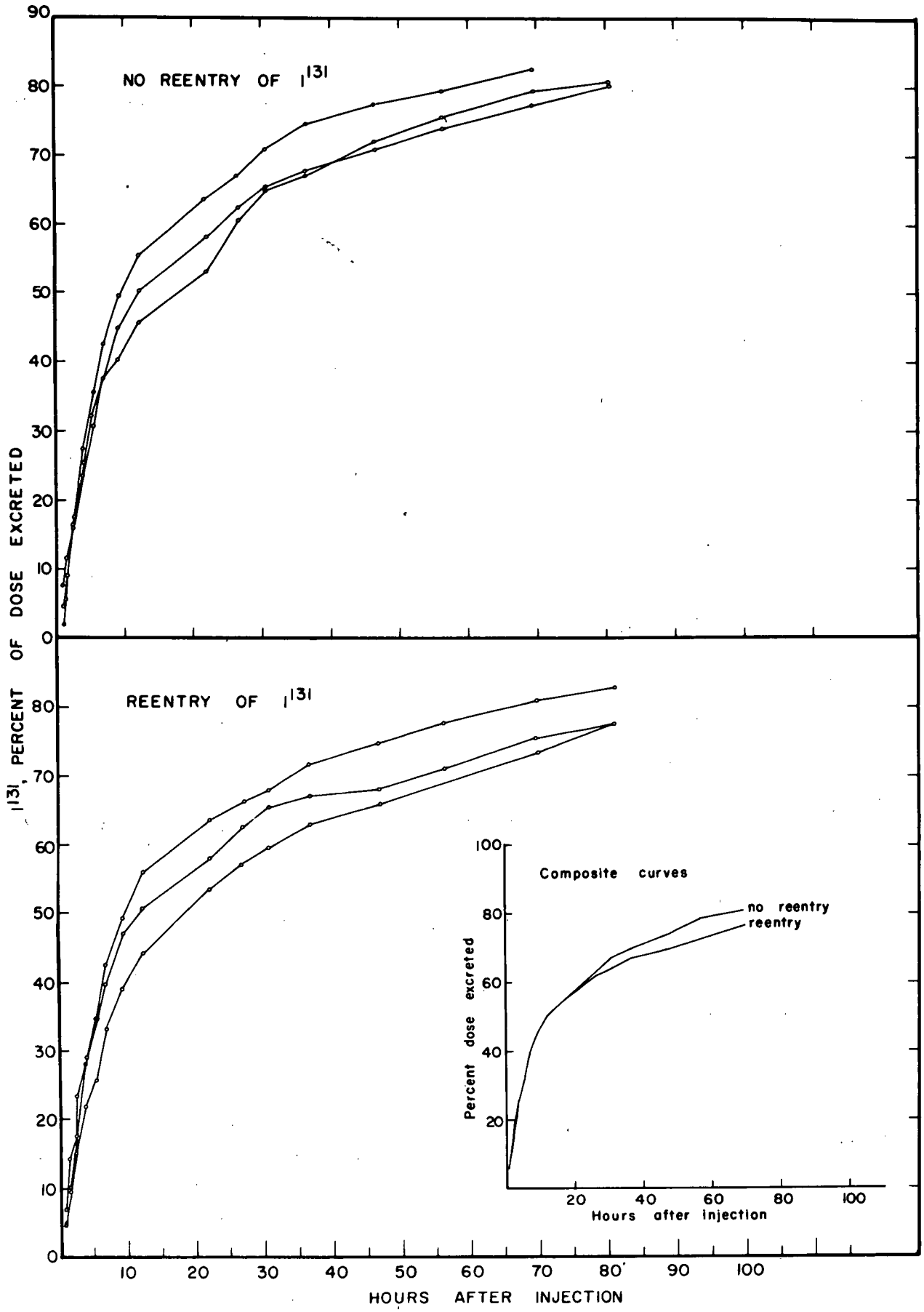
a. Effect of radioisotope reentry

Fish injected with a labeled substance excrete the isotope directly into the environment in which they live. Unless the water is continually renewed, the concentration of the isotope will increase in the ambient media and reenter the body of the fish. It was not possible to keep flounder in constantly renewed water, since experimental treatment necessitated controlling the salinity, temperature and elemental iodine content of the water. It was necessary therefore to assess the effect of I^{131} reentry on the rate of excretion of the isotope to determine whether normal behavior of the isotope in the body was upset by the effect.

Two groups of 3 flounder each were maintained in separate aquaria, each containing 7.5 liters of water, and under the same conditions of salinity ($19^{o}/oo$) and temperature ($18.0 \pm .3^{o}C.$). In the first group, the water was changed at very frequent intervals after injection of the fish to prevent any accumulation of excreted I^{131} in the water. Water was never changed in the second group of flounder to allow accumulation and reentry of the I^{131}. After injection, the disappearance of radioiodine from the bodies was followed by measuring the radioactivity of the entire living animal at intervals with an end-probe scintillation counter. The results are shown in Figure 18 with composite curves of the two groups shown in the inset. In this experiment, reentry of I^{131} did not become apparent in the excretion curves until 20 hours after injection.

The rapidity of which I^{131} reentry becomes apparent in I^{131} behavior in the body is a direct function of the rapidity of which excreted I^{131} accumulates in the surrounding water. This in turn is dependent on the volume of water in the experimental tank. In this experiment the three flounder in

Figure 18. Effect of reentry into the body of excreted isotope on the I^{131} excretion curves. Individual curves show the total excretion of a single dose of I^{131} from the bodies of 6 flounder (22.9 to 48.6 grams body weight). The three flounder shown by the upper curves were maintained in continually renewed sea water. The other three flounder shown by the lower curves were kept in unrenewed water. The effect of reentry first becomes apparent at about 20 hours after injection, as shown by inset composite curves. Experimental temperature 18.0 ± .3°C., salinity 19.0°/oo. June 7, 1958.


NO REENTRY OF I^{131}
REENTRY OF I^{131}
I^{131}, PERCENT OF DOSE EXCRETED
HOURS AFTER INJECTION
Composite curves
no reentry
reentry
Percent dose excreted
Hours after injection

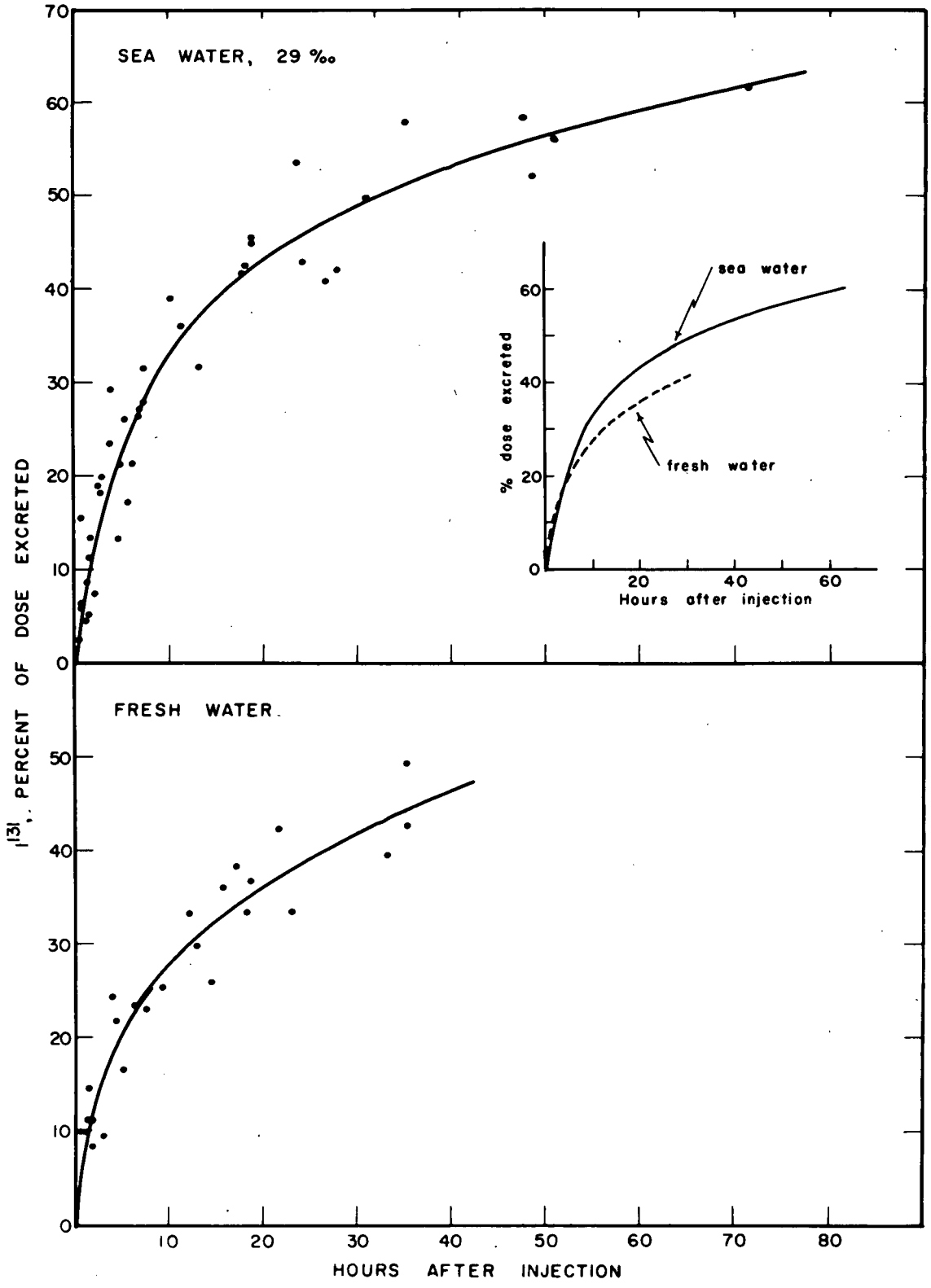
the reentry group were contained in 7.5 liters of sea water, or 2.5 liters per fish. In none of the experiments to be described were less than 2 liters of water per fish provided; usually the experimental tank contained 3 to 5 liters or more per fish. Reentry of excreted I^{131} was therefore without effect on the initial portions of the thyroid I^{131} uptake and blood I^{131} disappearance curves although measurements made after 36 to 48 hours probably contain some error in the form of heightened activity due to I^{131} reentry. Since only the events of the first few hours following injection are important for thyroid clearance calculations, return of excreted I^{131} into the body cannot be considered an important source of error.

b. Effect of salinity

For these experiments, carried out during February, 1958, small flounder weighing 2.5 to 20 grams were selected. They were maintained within 1°C. of the environmental temperature at the time of their capture in February (environmental temperature 6.8°C., experimental temperature 7.1 - 7.5°C.). The fresh water group were given a 5 day preadaptation to fresh water with added elemental iodine equivalent to the amount present in 30°/oo sea water - 43 µg. iodine per liter.

A comparison of the cumulative total excretion of I^{131} of fresh and salt water flounder is shown in Figure 19. Initially, fresh water flounder lose I^{131} at a much faster rate, but after the first few hours total excretion is more rapid in the salt water group. Since radioiodine is being trapped and held by the thyroid gland for long periods, this portion of the dose is essentially removed from the free inorganic iodide phase available for renal and extra-renal excretion. Thus the excretion curve is expected to reach an asymptote sometime before the whole dose is excreted. The difference between the asymptote and 100% excretion represents I^{131} trapped by the thyroid gland. Work on man (Keating, Power, Berkson and Haines, 1947) shows that I^{131} excretion

Figure 19. Effect of salinity on the cumulative total excretion of I^{131} by Platichthys stellatus. Each point represents the proportion of a standard dose of I^{131} excreted by an individual fish. Experimental temperature 7.3 ± .2°C. February, 1958.


SEA WATER, 29 ‰
FRESH WATER
I^{131}, PERCENT OF DOSE EXCRETED
HOURS AFTER INJECTION
% dose excreted
Hours after injection
sea water
fresh water

rates give reliable estimates of thyroid activity. The present experiment on fish was not extended long enough to determine where the curves plateau, though a real plateau may never be attained because of the slow turnover from the thyroid of organically bound I^{131} which will slowly be broken down. The inorganic I^{131} is returned to the iodide pool and finally excreted.

A comparison of the change in rate of radioiodide removal in fresh and salt water is shown in Figure 20. Rates were derived graphically from the eye-fitted cumulative excretion curves in Figure 19 and plotted with excretion rates as percentage of dose per hour on a log scale against time on a linear scale. During the first 2 hours after injection of salt water flounder, there is an initial very rapid fall in excretion rate. The change in rate then assumes a linear fall for several hours, which strongly suggests a simple exponential removal of I^{131} from a stable iodide compartment. Twenty hours after injection the rate of change (10.49% per hour)[1] decreases rather rapidly to another rate of 1.73% per hour, then decreases even further to an almost steady excretory rate at about 0.27% of dose per hour.

The excretion of I^{131} from fresh water flounder is distinctly different from that of salt water flounder (Fig. 19). The primary difference is that the fractional rate of change of excretory rate (Fig. 20) never assumes an exponential fall, but rather is continuously changing in a fashion that cannot be treated by the usual mathematical formulae for first-order reactions. The excretory rate at first is considerably above that of salt water flounder, but

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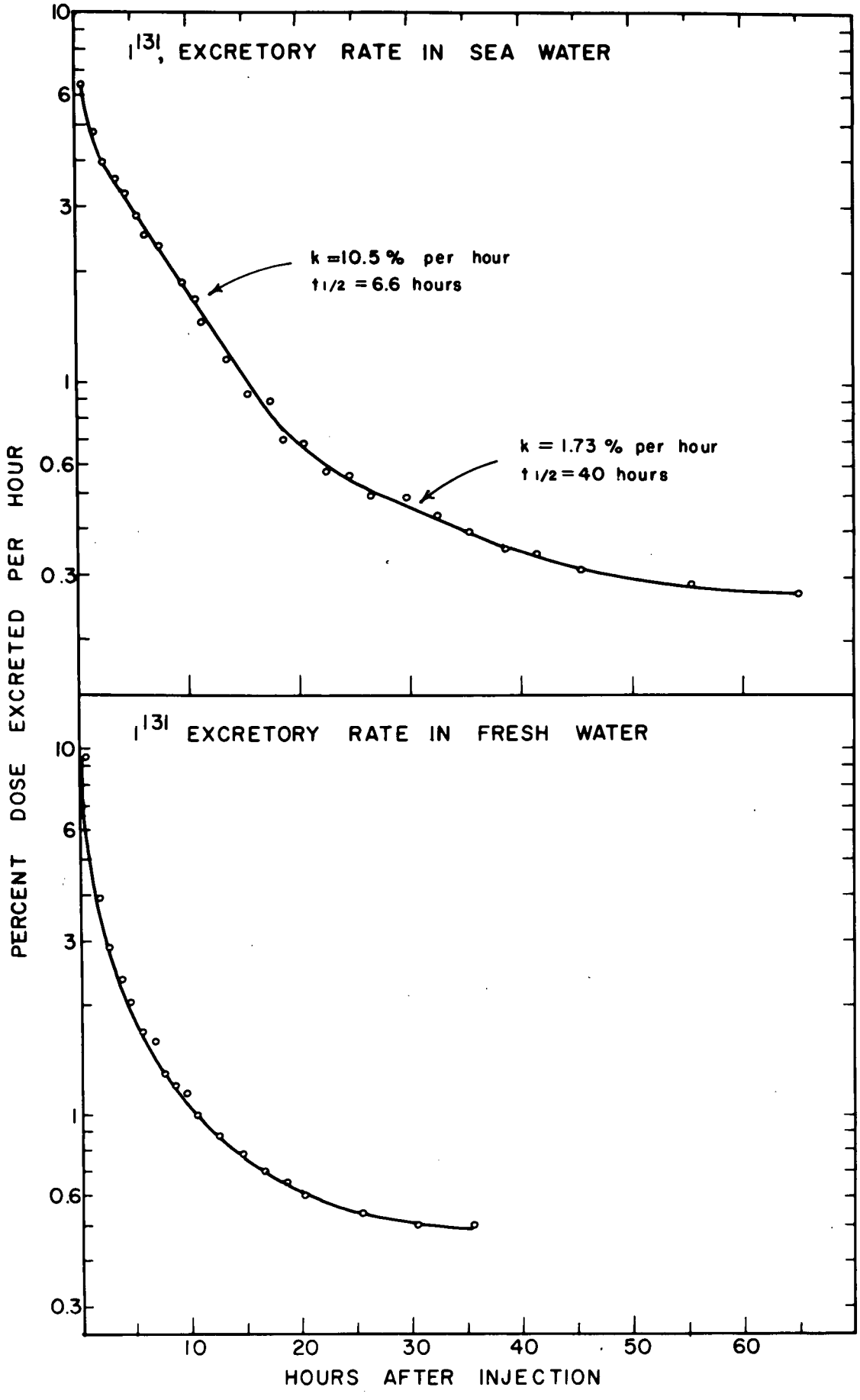
1. The rate constant k is the fractional rate of change of I^{131} with time and is calculated by the equation $I_1^* = I_o^* e^{-k}$ or $k = 1/t(\ln(I_1^* \text{ with } I_o^*))$ where I_1^* amount of I^{131} present at the time t and I_o^* amount of I^{131} present at zero time. The half-value time, $t\,\frac{1}{2}$, representing the time for removal of half the I^{131} present is calculated by:

$$t_{\frac{1}{2}} = \frac{2.3 \log \frac{1}{2}}{k} = \frac{0.693}{k}$$

Figure 20. Rate of removal of I^{131} from the bodies of Platichthys stellatus. Rates were derived graphically from the fresh water and salt water excretion curves shown in Figure 27 by the relation: $I_1^* = I_0^* e^{-k}$ where I_1^* = amount of I^{131} present at time t and I_0^* = amount of I^{131} present at zero time.


I^{131}, EXCRETORY RATE IN SEA WATER
k =10.5 % per hour
t 1/2 = 6.6 hours
k = 1.73 % per hour
t 1/2 = 40 hours
I^{131} EXCRETORY RATE IN FRESH WATER
PERCENT DOSE EXCRETED PER HOUR
10
6
3
1
0.6
0.3
10
20
30
40
50
60
HOURS AFTER INJECTION

falls very rapidly, so that between the first and twenty-fifth hour, the excretory rate of I^{131} is less in fresh than in salt water. At 35 hours after injection, however, the rate appears to level at 0.5% of dose per hour, at which point the experiment was terminated.

Since the fractional rate of change of I^{131} excretion rate with time does not proceed as a logarithmic function, it can only be assumed that the volume of distribution of the tracer dose (radioiodide space) never stabilized during the experiment. The excretory organs, primarily the kidney and gills, presumably do not distinguish between I^{131} and I^{127}. Assuming equilibrium of the tracer dose in the body fluids, the proportion of the dose excreted is also the proportion of all the iodide in the body which is excreted. However, if the I^{131} dose does not rapidly come into equilibrium with the body fluids, but continues to penetrate slowly, there will result a continual enlargement of the volume of distribution of the I^{131}. This appears to be the case in the fresh water flounder and to some extent in salt water flounder. While the true reason for these observations must await further research, a probable explanation is that the adaptation time of the flounder to fresh water was inadequate to ensure arrival at a stable level of osmotic regulation. It was shown in a previous section (Fig. 1a) that the osmotic concentration of the blood of starry flounder transferred abruptly to fresh water dropped appreciably but reached an essentially stable level within 3 days following transfer. These experiments were carried out at 15°C., whereas the present studies on thyroid activity were carried out in February at a much lower temperature (7.1 - 7.5°F.), which very probably slowed the arrival of the body fluids at osmotic equilibrium in the new environment. This hypothesis is given considerable support by an examination of the disappearance rate of radioiodine from the blood in the same group of fresh water flounder. As shown in Figure 23 the concentration of I^{131} in the blood decreases hardly at all between the third and ninth hour following injection, even though

10.5% of the injected dose was excreted during this period (Fig. 19). The most plausible explanation is that the volume of distribution of the injected I^{131} has shrunk! This is exactly what would be expected if the measurements were made during the period that the body fluids were shifting toward a new level of stable osmoregulation. These events were shown in Figure 4. It was pointed out that a decrease in the extracellular space (essentially the same as the iodide space) was frequently observed in flounder after transfer to fresh water from sea water, an effect apparently caused by a shift of water from without to within the cells. Whatever the exact cause may be, a decrease in the volume of I^{131} distribution seems a probable explanation for both the very slow disappearance rate of radioiodide from the blood and the gradual decrease in the fractional rate of change of I^{131} excretion rates.

c. Relative importance of renal and extrarenal excretion of I^{131}

Excretory clearances of I^{131} calculated from the same data for the first 5 hours after injection are given in Table XII. These clearance estimates represent the proportion of the radioiodide space which is cleared per hour by the organs of excretion. Although usually presented as the volume of blood or plasma cleared, it is understood that the total volume of inorganic iodide is being cleared by the kidney or gills or other pathway of removal.

As expected from the slower excretory rates (Fig. 20) in the fresh water flounder, excretory clearances also are less in the latter than in salt water flounder. Averages of the clearance estimates, omitting the first hour when the concentration of I^{131} in the blood is changing rapidly during absorption and distribution of the dose, give 0.242 grams of blood in the salt water flounder and 0.141 grams of blood in the fresh water flounder. Thus, in spite of the greater urine flow of the fresh water fish, total I^{131} removal is less rapid

Table XII

Excretory clearance of radioiodine from the blood of flounder in salt and fresh water. Clearances adjusted for body weight and expressed as grams of blood cleared of radioiodine per hour in a 10 gram flounder.

Salt Water Starry Flounder

Hour	% dose excreted per hour	Blood concentration % dose per gram x Body weight/100	Clearance
1	5.4	2.25	0.24
2	4.3	1.8	0.238
3	3.7	1.43	0.258
4	3.3	1.36	0.242
5	3.0	1.29	0.232

Fresh Water Starry Flounder

Hour	% dose excreted per hour	Blood concentration % dose per gram x Body weight/100	Clearance
1	4.8	2.45	0.196
2	3.3	2.45	0.134
3	2.55	1.51	0.169
4	2.12	1.51	0.141
5	1.8	1.48	0.121

Direct data are not available to indicate the relative importance of the several possible pathways of exchange of iodide with the external environment in either fresh or salt water. However, it is possible to make a rough estimate of the volume of urine needed to remove all of the I^{131} being excreted assuming that there is no extrarenal excretion of any kind. By comparing these hypothetical urine flows with average urine flow values for fresh and salt water

species reported in the literature, one arrives at a rough indication of the proportion of I^{131} removed by renal and extrarenal pathways. Urine flows were calculated by dividing the proportion of the dose excreted per hour by the urinary concentration of I^{131} expressed as the biological concentration coefficient (% dose per gram of urine x body weight / 100). The calculated values are summarized in Table XIII. These values are far above actual urine flow measurements reported in the literature (summarized in Table III of Black's review paper, 1957). Values reported for salt water species range from about 3 to 30 ml. of urine per kg. body weight per day as compared to the average of 118.7 ml./kg./day calculated for salt water flounder assuming all the radioiodide was excreted renally. Similarly, reported urine flows for freshwater species range between 7 and 106 ml./kg./day as compared to the 623 ml./kg./day average urine flow calculated for the fresh water flounder. It is evident that a very large proportion of the excreted I^{131} is being removed extrarenally in both salt and fresh water flounder.

The possible pathways of extrarenal iodide removal are the gills, the mucous membranes of the mouth and with the fecal wastes. In man only minute quantities of iodide are lost extrarenally, that is, in the feces (Nelson, et.al., 1947; Keating and Albert, 1948) the expired air and the perspiration (Riggs, 1952). In fishes it is also doubtful that appreciable amounts of iodide leave in the feces, particularly in fasted animals such as were used in these experiments. Hence, the gills and mucous membranes must constitute the major organs of extrarenal iodide exchange.

Measurements of the concentration of I^{131} in the gill lamellae of flounder in both fresh and salt water were undertaken in the hope of providing an indication of the relative importance of the gills in iodide exchange. The results are given in Table XIV. With the exception of the first 6 hours in the case of fresh water flounder, the radioiodide concentration is appreciably higher in the gill lamellae than in the blood, indicating a cellular concentra-

Table XIII

Calculated urine flows of individual fresh and salt water flounder, assuming no extrarenal excretion of I^{131}. Urinary concentration expressed as % of dose per gram of urine x body weight/100.

Salt Water Flounder

Time	Urinary Concentration	% dose excreted per hour	Calculated urine flow ml./100 gm./hr.	ml./kg./day
3:52	3.73	3.2	0.9005	225
4:55	6.05	3.25	0.496	119
6:03	4.99	2.43	0.537	129
7:00	3.42	2.1	0.701	168
7:05	4.91	2.15	0.489	117
7:20	5.92	2.0	0.392	94
10:00	2.8	0.8	0.282	67.7
24:25	4.42	0.35	0.124	29.7
			Average	118.7

Fresh Water Flounder

Time	Urinary Concentrated	% dose excreted per hour	Calculated urine flow ml./100 gm./hr.	ml./kg./day
12:10	0.504	1.35	.90	427
13:00	0.388	1.88	.85	525
18:11	0.232	0.95	.66	681
23:00	0.35	0.73	.57	391
35:35	0.11	0.50	.50	1090
			Average	623

tion of the isotope. The interpretation, however, is difficult, for it is impossible to say whether the iodide is being concentrated in the gills prior to secretion to the exterior media or whether it represents simply a cellular accumulation with no net movement in either direction. Marine flounder may secrete the univalent iodide ions together with univalent sodium and chloride ions via the "chloride secreting cells" to the exterior. If so, a concentration of iodide in the gills is not surprising. Fresh water flounder are not expected normally to secrete iodide, since its concentration is far lower in fresh water than in the blood. There may possibly exist an active iodide absorption mechanism in the gills of fresh water flounder (hence the high concentrations of radioiodide in the gill lamellae), but Krogh (1938) found that no such mechanism existed in goldfish and that iodide was lost slowly from the body by diffusion. It should be possible to quantitate experimentally the relative proportions of iodide transferred by each of the pathways of exchange with external environment.

Table XIV

The concentration of I^{131} in the gill lamellae of starry flounder expressed as percentage of the concentration of I^{131} in the blood. Body weight 24 to 105 grams.

Hours after injection	Fresh water flounder	Average	Salt water flounder (25°/oo sea water)	Average
1	83.6	83.6	——	——
3	40.4, 88.3	64.3	109, 267	188
6	61.7, 69.2	65.4	83.4, 108	95.7
12	147	147	123, 214	168.5
24	210, 309	259.5	79, 157	118
48	264, 474	369	133, 175	154
100	208, 212	210	128, 175.5, 202	168.5

d. Effect of size

When a number of starry flounder of all sizes are simultaneously injected with tracer iodide, killed together several hours later and measurements made of the proportion of the dose excreted, it is seen that small flounder lose the isotope more rapidly than large flounder. An example is shown in Figure 21a, in which the percentage of dose excreted is plotted against body weight for three sampling periods after injection.

The points best conformed to a linear relationship with body weight placed on a log scale. The results of each of three salinity treatments - 46°/oo, 25°/oo and fresh water - were plotted on semilogarithmic paper and fitted with a straight line by the method of least squares. From each of these lines of best fit, three points were graphically derived representing the total excretion of flounder weighing 5, 15 and 50 grams and plotted against time after injection (Fig. 21b). In all three salinities the tracer dose of I^{131} is more rapidly excreted by small fish. Since the body does not distinguish between stable and radioactive iodide and since the intake of iodide is assumed to balance its loss from the body, it is evident that the net turnover of iodide is greater in small than in large flounder. The relationship between body size and rapidity of iodide exchange is yet another indication of the more intensive metabolic rate of small flounder.

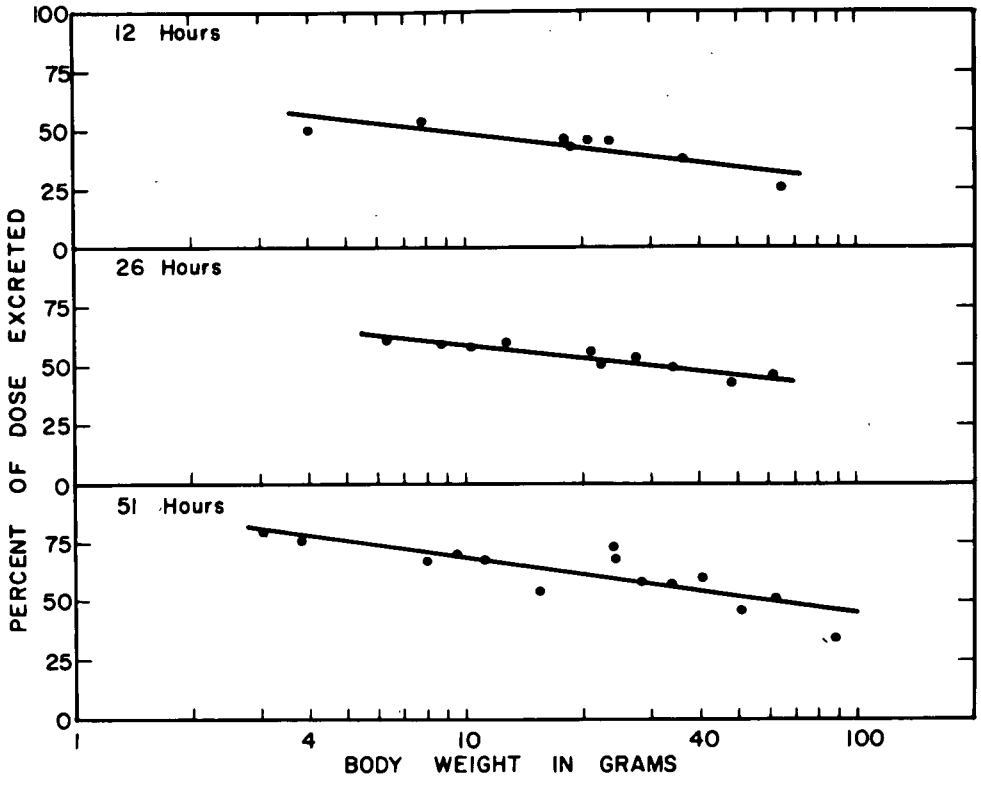
2. Factors Influencing Uptake of Radioiodide By the Throid Gland

a. Effect of iodide content of the water

The effect of the quantity of elemental iodide in the ambient water on the uptake of radioiodine by the thyroid is shown in Figure 22. It is apparent from Figure 22a that the rate of accumulation of I^{131} by the thyroid of flounder adapted to fresh water is much greater in natural fresh water than in fresh water reinforced with 50 ug iodine per liter (40 ug iodine as KIO_3 and 10 ug iodine as KI),

Figure 21a. Effect of body size on the excretion of I^{131} from Platichthys stellatus in sea water of 25 °/oo. Each point represents the proportion of a standard dose of I^{131} excreted by an individual fish at the indicated time after injection. Points fitted by the method of least squares. Experimental temperature 15.0 ±.2°C.

Figure 21b. Effect of body size on the excretion of I^{131} from Platichthys stellatus in fresh water, normal sea water and concentrated sea water. The three points for each curve representing 5, 15 and 50 gram flounder were graphically derived from lines of best fit through individual total excretion values as shown in Figure 21a. Experimental temperature 15.0 ±.2°C.


12 Hours
26 Hours
51 Hours
PERCENT OF DOSE EXCRETED
100
75
50
25
0
1
4
10
40
100
BODY WEIGHT IN GRAMS

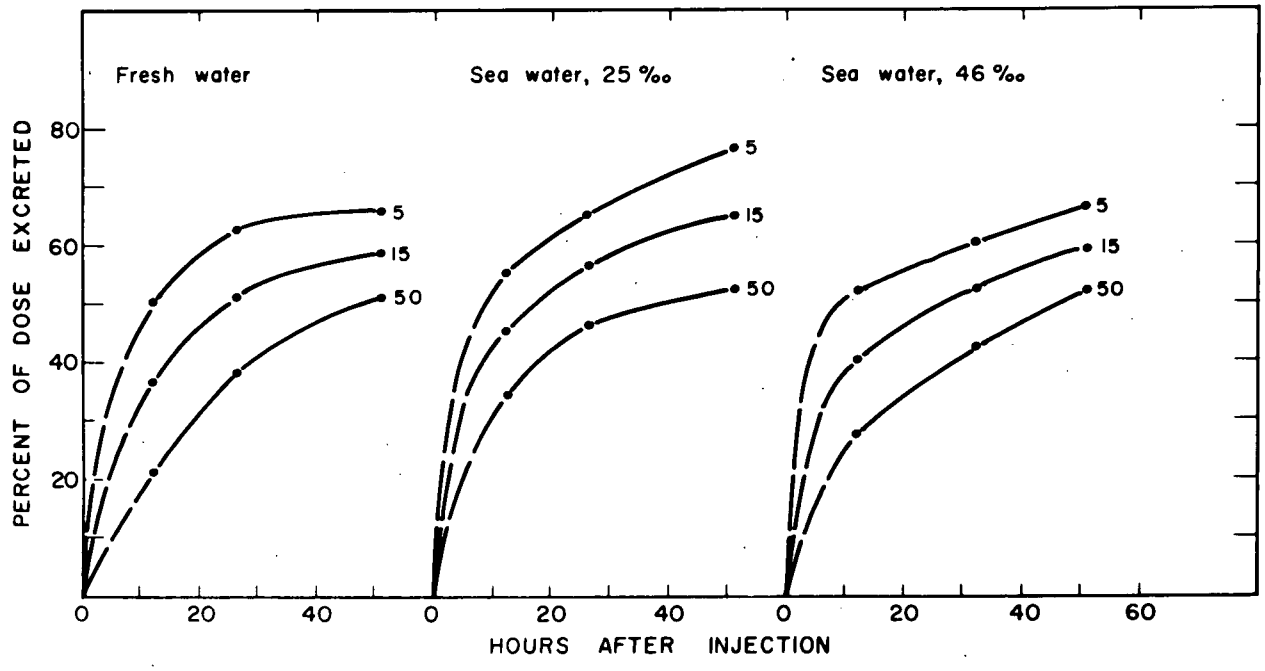
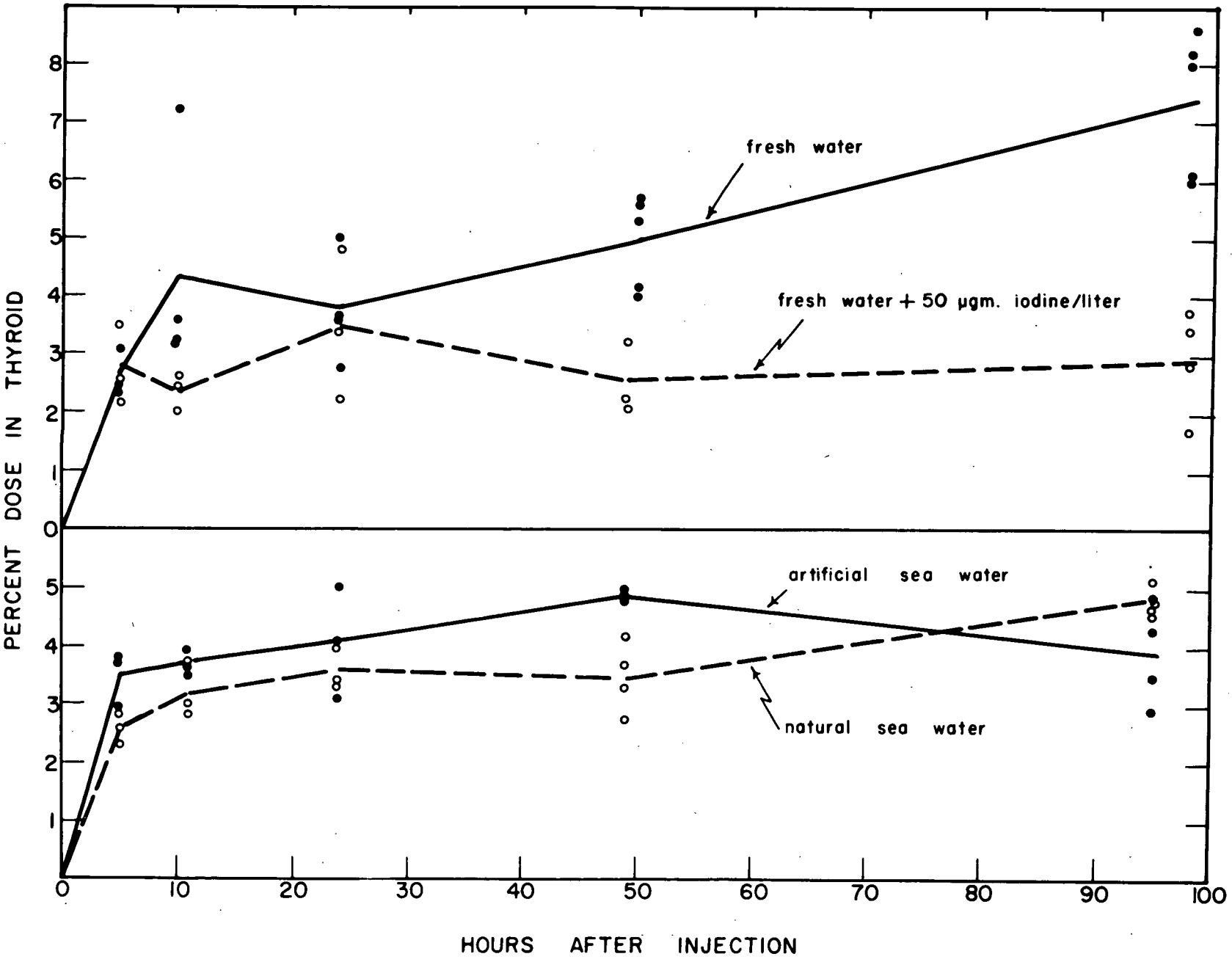

Fresh water
Sea water, 25 ‰
Sea water, 46 ‰
PERCENT OF DOSE EXCRETED
80
60
40
20
5
15
50
0
20
40
60
HOURS AFTER INJECTION

Figure 22. Effect of elemental iodine content of the water on thyroid I^{131} uptake of Platichthys stellatus.

22a. Individual thyroid uptakes of flounder in fresh water are shown by solid circles and the average by the solid line. Uptakes of flounder in iodine reinforced water are shown by open circles with a broken line average. Uptake is stimulated in iodine deficient fresh water.

22b. Flounder in iodine deficient sea water have greater uptakes (solid circles, solid line average) than do flounder in natural sea water which contains about 50 ugm. iodine/liter (open circles, broken line average).


PERCENT DOSE IN THYROID
HOURS AFTER INJECTION
fresh water
fresh water + 50 µgm. iodine/liter
artificial sea water
natural sea water
0
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70
80
90
100

the normal iodine content of undiluted sea water (Strickland, J.D.H., 1956, pers. comm.).

To assess the effect of low iodine in salt water, artificial sea water was prepared following the formula of Brujewicz (cited in Table 37, Sverdrup, Johnson and Fleming) using analytical grade reagents. The solution contains the major elements found in sea water (chloride, sodium, magnesium, calcium, potassium, bromide, sulphate and bicarbonate) without iodine. However, iodine undoubtedly is present in small amounts as a contaminant in the reagents used, so that the prepared solution cannot be considered iodine free. As shown in Figure 22b, thyroidal uptake of radioiodine by starry flounder is greater in iodine deficient artificial sea water than in natural sea water of the same salinity (30°/oo).

The increased rate of thyroidal accumulation of radioiodine by flounder in fresh water and artificial sea water as compared to flounder in iodine reinforced fresh water and natural sea water are both interpreted as the familiar compensatory response of the iodide trapping mechanism to iodine deficiency. As already discussed in the introductory remarks to this section, thyroid hyperplasia as a result of iodine deficiency in the water has been demonstrated in fish by several authors. It is evident that the quantity of iodine available to a fish is an extremely important factor influencing the validity of the radioiodine uptake method as a measure of thyroid activity. Low levels of iodine in the environment produce a hyperactivity of the "iodide trap" resulting in a high rate of radioiodide accumulation that does not represent an overall increase in release of hormone into the blood stream. This effect is of course particularly important in comparing thyroid activity of flounder in fresh water containing less than one microgram per liter and in sea water containing about 50 micrograms per liter (at salinity 35°/oo). For this reason, fresh water was always reinforced with iodide to bring its iodide concentration equivalent to sea water.

b. Effect of salinity

In the foregoing section it was shown that the thyroid uptake of I^{131} by flounder adapted to fresh water is greater than that of marine flounder, but that essentially all of the difference was due to iodine deficiency in fresh water. With elemental iodide added to the fresh water, both fresh water and sea water flounder were found to have about the same thyroidal uptakes.

From the initial efforts to separate differences in thyroid activity of starry flounder in different salinities, it became apparent that thyroidal uptake of I^{131} was inadequate as a criterion for activity evaluation for two reasons: a) the method is insensitive and cumbersome because of the rather large variability between individual fish in the percentage uptake of I^{131} by the thyroid, necessitating large sample sizes for each test, and b) the validity of the method rests on the assumption that the disappearance rate from the blood of a single injected dose of I^{131} is uninfluenced by salinity. It has already been pointed out that there was good reason to doubt this last assumption because of marked differences in electrolyte behavior and the different excretion rates of radioiodide between fresh water and salt water fish. For these reasons, thyroidal clearance of radioiodide from the blood has been used instead of thyroid uptakes to evaluate thyroid activity.

For the determination of thyroidal clearance of I^{131} from the blood, simultaneous measurements of the rate of increase of concentration of I^{131} in the thyroid and the I^{131} concentration in the blood are taken. This was done by injecting standard tracer doses of I^{131} into the body cavities of two groups of flounder, one group maintained in sea water and the other previously adapted to iodine-reinforced fresh water. Individual fish were sacrificed at intervals of time after injection and the thyroid and a blood sample removed from each for counting. The results are given in Figure 23, where the solid circles represent the thyroidal I^{131} uptakes and the open circles the blood I^{131} con-

centration as the biological concentration coefficient.

i. The behavior of radioiodide in the blood

Figures 23 and 24 show that several components are present in the blood I^{131} curves. In both fresh and salt water flounder, the concentration in the blood increases rapidly after the intraperitoneal injection as the dose is absorbed into the blood stream. The rate of absorption differs considerably in the two groups and will be discussed separately.

Marine Flounder

In small marine fish (2.5 - 20 grams), absorption from the body cavity is rapid (Fig. 23). The blood appears to reach a peak concentration of nearly 5.5% of dose[1] at one-half hour after injection, followed by a rapid fall to about 2.2% within a few minutes. This rapid rise and fall is interpreted as a rapid absorption of I^{131} into the blood stream via the vascular peritoneum followed by a less rapid diffusion into the extracellular space or, more accurately, the iodide space. At one hour after injection, the disappearance rate becomes exponential. Between the first and third hours the disappearance rate has a half-value time ($t_{\frac{1}{2}}$) of 3.45 hours (D = 20%/hour) but decreases after 3 hours to a slower rate of removal ($t_{\frac{1}{2}}$ = 12.8 hours, K = 5.4%/hour). The reason for the change in disappearance rate at 3 hours is not known. In mammals, the removal of radioiodine from the iodide space remains exponential until the appearance of plasma-bound radioiodine in the blood causes a gradual decrease in the apparent removal rate (McConahey, Keating and Power, 1949; Riggs, 1952). Because of the relatively low activity and iodine turnover rate of the teleost thyroid it is very unlikely that plasma-bound radioiodine appears in appreciable amounts until

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1. Blood I^{131} concentration is calculated as the percentage of administered dose per gram of blood x body weight/100 (biological concentration coefficient), but for brevity in this discussion blood I^{131} concentration will be expressed simply as percentage of dose.

Figure 23. Effect of salinity on thyroid I^{131} uptake and blood I^{131} disappearance rate of Platichthys stellatus. Thyroid uptake ( ) is expressed as percentage of dose (5 microcuries carrier-free NaI^{131} per fish, intraperitoneal injection) accumulated by the whole gland; blood I^{131} is expressed as percentage of dose per gram of blood x body weight/100. I^{131} is concentrated more rapidly in the thyroids of sea water flounder in spite of the more rapid disappearance of I^{131} from the blood. Salinity of sea water 29 °/oo; fresh water reinforced with 40 micrograms/ liter of iodine. Experimental temperature 7.5 ±.3°C.

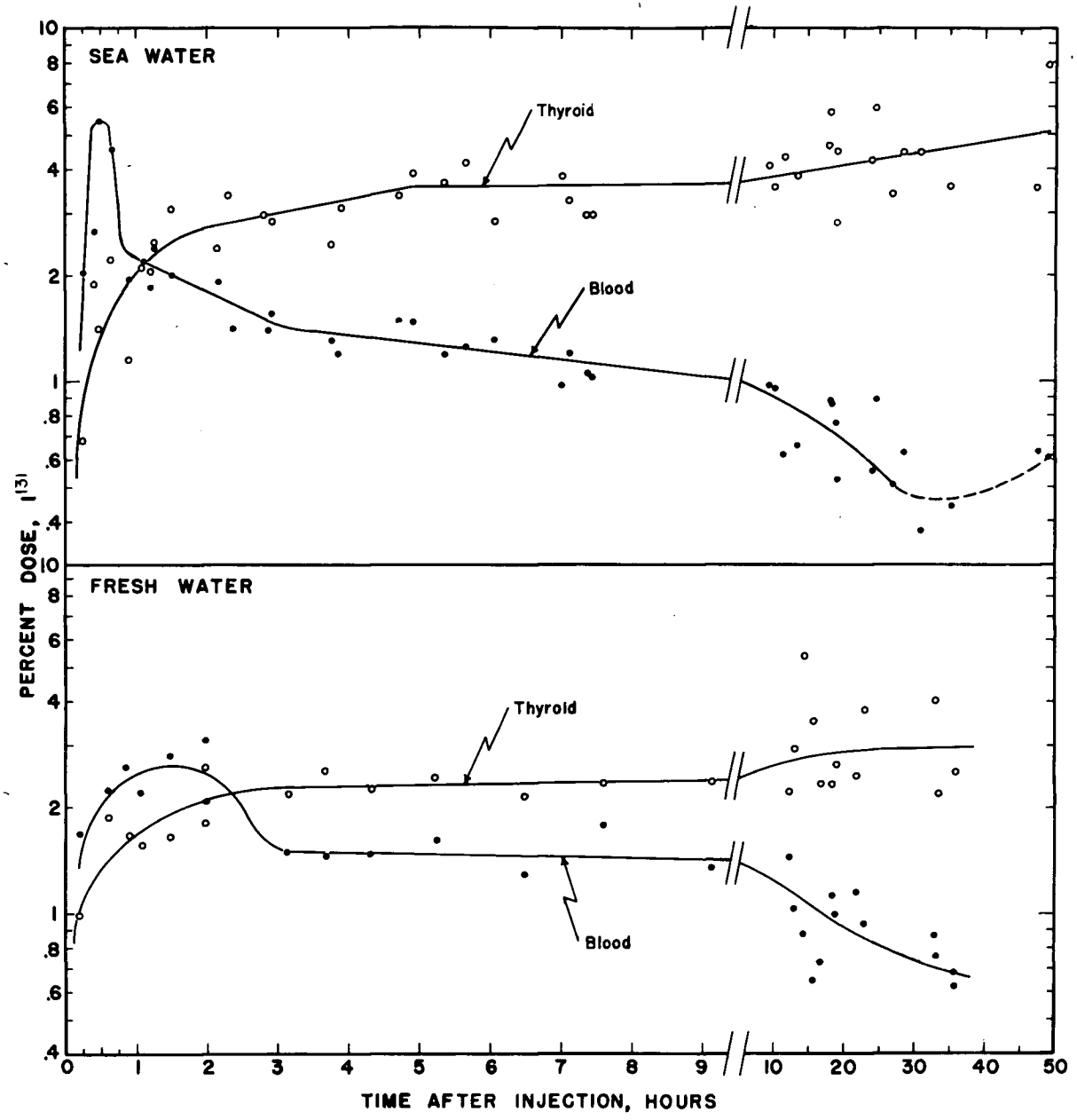
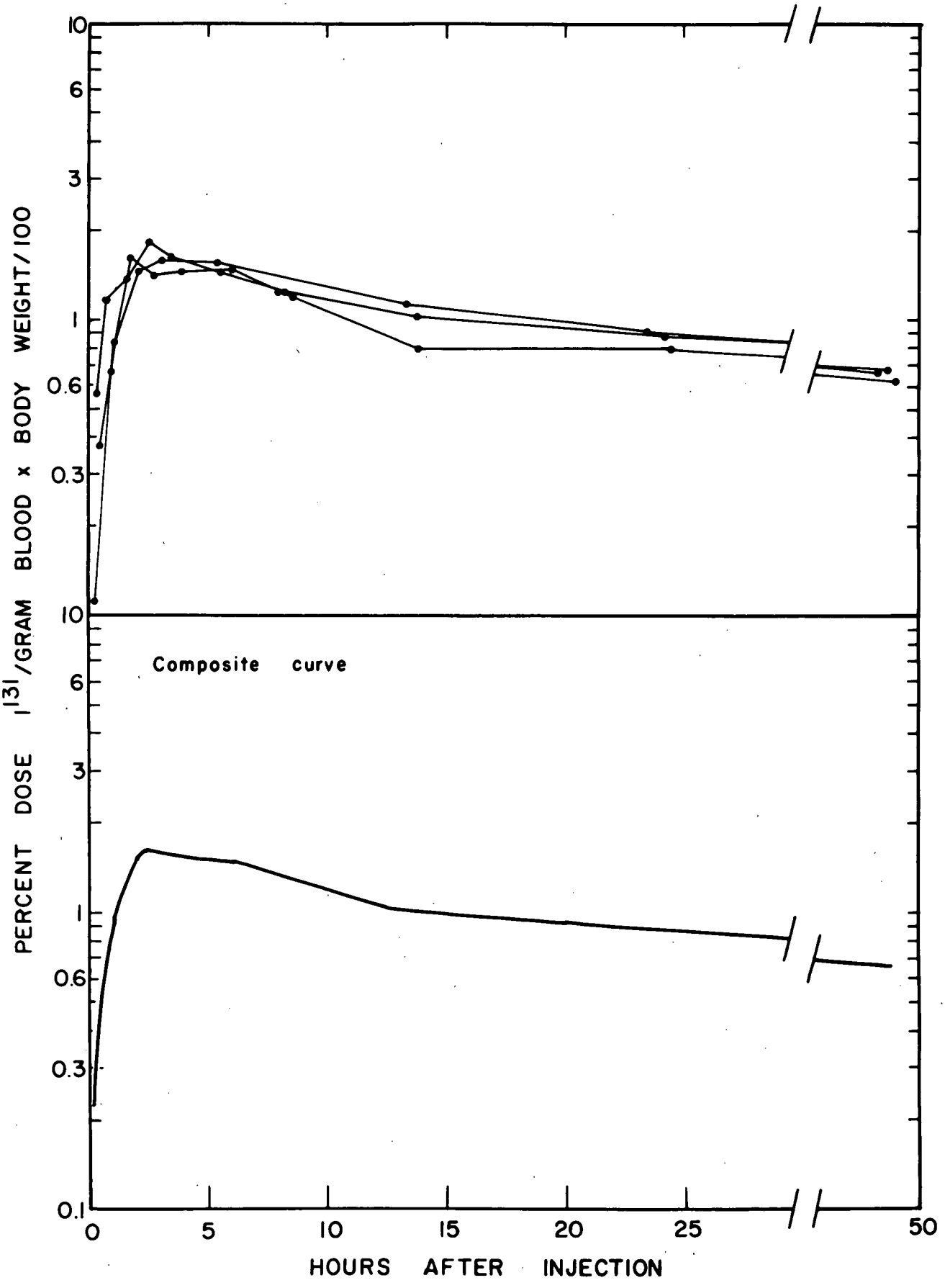

SEA WATER
Thyroid
Blood
FRESH WATER
Thyroid
Blood
PERCENT DOSE, I(131)
TIME AFTER INJECTION, HOURS
10
8
6
4
2
1
.8
.6
.4
0
1
2
3
4
5
6
7
8
9
10
20
30
40
50

Figure 24. Behavior of radioiodine in the blood of 3 large (102 to 213 gm.) Platichthys stellatus in sea water. Blood serially sampled at intervals from the caudal artery. A composite curve of the three individual curves is shown. Salinity 29 °/oo, experimental temperature 7.5 ±.3°C.


10
6
3
1
0.6
0.3
10
6
3
1
0.6
0.3
0.1
Composite curve
PERCENT DOSE I^{131}/GRAM BLOOD x BODY WEIGHT/100
0
5
10
15
20
25
50
HOURS AFTER INJECTION

at least 24 hours after injection of a tracer dose. It is also very doubtful that reentry of excreted I^{131} into the body from the ambient water can be cause for the change in rate of I^{131} removal (Fig. 18). It seems probable that some change has occurred in the volume of distribution of the I^{131} in the body fluid, the explanation of which must await further experimentation.

Serial sampling of blood was carried out on three large marine flounder over a period of nearly 50 hours (Fig. 24). Absorption of the dose from the coelom is considerably less rapid than in the small flounder and I^{131} appears not to enter the blood stream more rapidly than it diffuses into the iodide space since its concentration does not reach the high levels seen in small flounder. The slower absorption of I^{131} into the blood stream appears to be another example of the lower metabolic rate of large flounder. It will be recalled that disappearance rate of iodine from the body of flounder was more rapid in small than in large flounder, indicating a more rapid body turnover of elemental iodine in smaller individuals.

Disappearance of radioiodine from the blood is slower in large than in small flounder. After equal diffusion of the I^{131} with the body fluids at about 6 hours after injection, it assumes an exponential removal curve with a half-time of 11.95 hours (K = 5.8%/hour) as calculated from the composite curve in Figure 24. About 13 hours after injection, the rate of removal slows to a half-time of 52.8 hours (K = 1.3%/hour). As expected, because of the slower I^{131} total excretion from the bodies of large flounder, the removal of I^{131} from blood of large flounder is less rapid than from the blood of small flounder.

The upswing of the blood I^{131} concentration curve of small flounder at 40 to 50 hours after injection may indicate the appearance of plasma-bound radioiodine in the blood at this time. By this time I^{131} which has been trapped by the thyroid will have been synthesized into hormonal iodine, released into the blood stream and bound with plasma proteins. In man, protein-bound

iodine appears in the blood within a few hours after injection (McConahey, Keating and Power, 1949), particularly in hyperthyroidism. No measurements appear to have been made of plasma-bound iodine among the fishes, but its appearance in the blood is probably very slow because of the relatively slow uptake and release of radioiodine by the thyroid gland.

Fresh Water Flounder

The absorption of radioiodine from the body cavity of small fresh water flounder is much slower than in small marine flounder (Figure 23). After diffusion throughout the iodide space the I^{131} disappears from the blood at an extremely slow rate ($t_{\frac{1}{2}}$ = 63.6 hours, K = 1.09%/hour)., a slower rate than would be expected from the total excretion of I^{131} (about 10.5% of the dose between the third and ninth hours after injection, (Fig. 19). The excretion of the I^{131} dose in fresh water flounder has already been discussed in a previous section, where it was postulated that the volume of I^{131} distribution in these flounder decreased somewhat after injection indicating that the body fluids were still shifting toward a new stable level of osmoregulation after transfer to fresh water. It is emphasized that such a change in the volume of the iodide space is without effect on the thyroid clearances of I^{131} from the blood since this function depends on the activity of the "iodide trap" of the thyroid follicles and the level of I^{131} in the blood passing through the gland at any one time.

ii. The thyroidal clearance of radioiodide from the blood

In Table XV, thyroidal clearance of I^{131} from the blood of fresh water and salt water flounder are calculated from the data shown in Figure 23 for the period when the uptake of I^{131} by the thyroid and removal of I^{131} from the blood were both nearly exponential. It is apparent that the average thyroid clearance of I^{131} in fresh water flounder (about .00135) is several times less

than the average clearance calculated for salt water flounder (about .0197). Since the level of elemental iodine in the environment was the same for both groups, these results appear to be conclusive evidence that thyroid activity is greater in salt water flounder than in fresh water flounder. The experiment from which these values were derived (Fig. 23) is a replicate of an earlier experiment which also showed much greater clearance rates for salt water flounder.

Table XV

Thyroid clearance of radioiodide from the blood of Platichthys stellatus. Clearance rates expressed for a 10 gram flounder as grams of blood cleared of I^{131} per hour. Clearance rates were calculated from the data shown in Figure 23 during the period of exponential disappearance of I^{131} from the blood by the formulae:

$$\frac{\text{rate of } I^{131} \text{ uptake by the thyroid}}{\text{mean blood concentration of } I^{131}}$$

Fresh Water Flounder

Hours after injection	Average blood I^{131} concentration	Thyroid uptake	Clearance
3 - 4	1.500	.02	.00133
4 - 5	1.485	.02	.00135
5 - 6	1.470	.02	.00136

Salt Water Flounder

Hours after injection	Average blood I^{131} concentration	Thyroid uptake	Clearance
2 - 3	1.6	.27	.0169
3 - 4	1.38	.28	.0202
4 - 5	1.32	.29	.022

c. Effect of body size on thyroid activity of starry flounder and speckled sand dab

Starry Flounder

The effect of body size on thyroid activity was assessed by injecting a large number of flounder of variable body size with a standard dose of I^{131} and sampling groups of fish at predetermined intervals of time after injection. Thyroid samples were removed, prepared for counting, and their activity measured with the well-crystal scintillation counter, thereby avoiding self-absorption problems due to variable sample mass.

In Figure 25 an example is shown of the effect of body size on thyroid I^{131} uptake of flounder in sea water of 25°/oo. The diphasic eye-fitted line placed through the individual thyroidal uptakes is included only to indicate a trend between uptake and body size and is not to be interpreted as having high significance in itself. Uptakes are relatively high in small flounder of 2 to 6 grams, are much less in 20-30 gram flounder and increase again with increasing body size above 30 grams. The same interaction between body size and thyroidal uptakes was equally prominent in fresh water adapted flounder and in flounder adapted to concentrated sea water (45°/oo).

Since I^{131} is excreted more rapidly from the body in small than in large flounder (Figs. 21a and 21b), the isotope is also disappearing more rapidly from the blood stream. Hence, thyroidal trapping of I^{131} by small flounder is proceeding against a lower concentration of I^{131} in the blood than in large flounder. This means that the actual thyroid activity of a 2 gram flounder is even greater than indicated by the measured uptakes as compared to the activity of a 20 gram flounder. For the same reason, some small part of the measured increase in thyroid I^{131} uptake of larger flounder (100-200 grams) may be due to the slower excretion of I^{131} in these individuals.

Speckled Sand Dab

The effect of body size on thyroid I^{131} uptake of sand dab is shown in

Figure 25. Effect of body size on thyroid activity of Platichthys stellatus. Indicated times represent hours after injection of a tracer dose of I^{131}. Lines fitted by eye. Experimental temperature 15°C., salinity 25 °/oo.

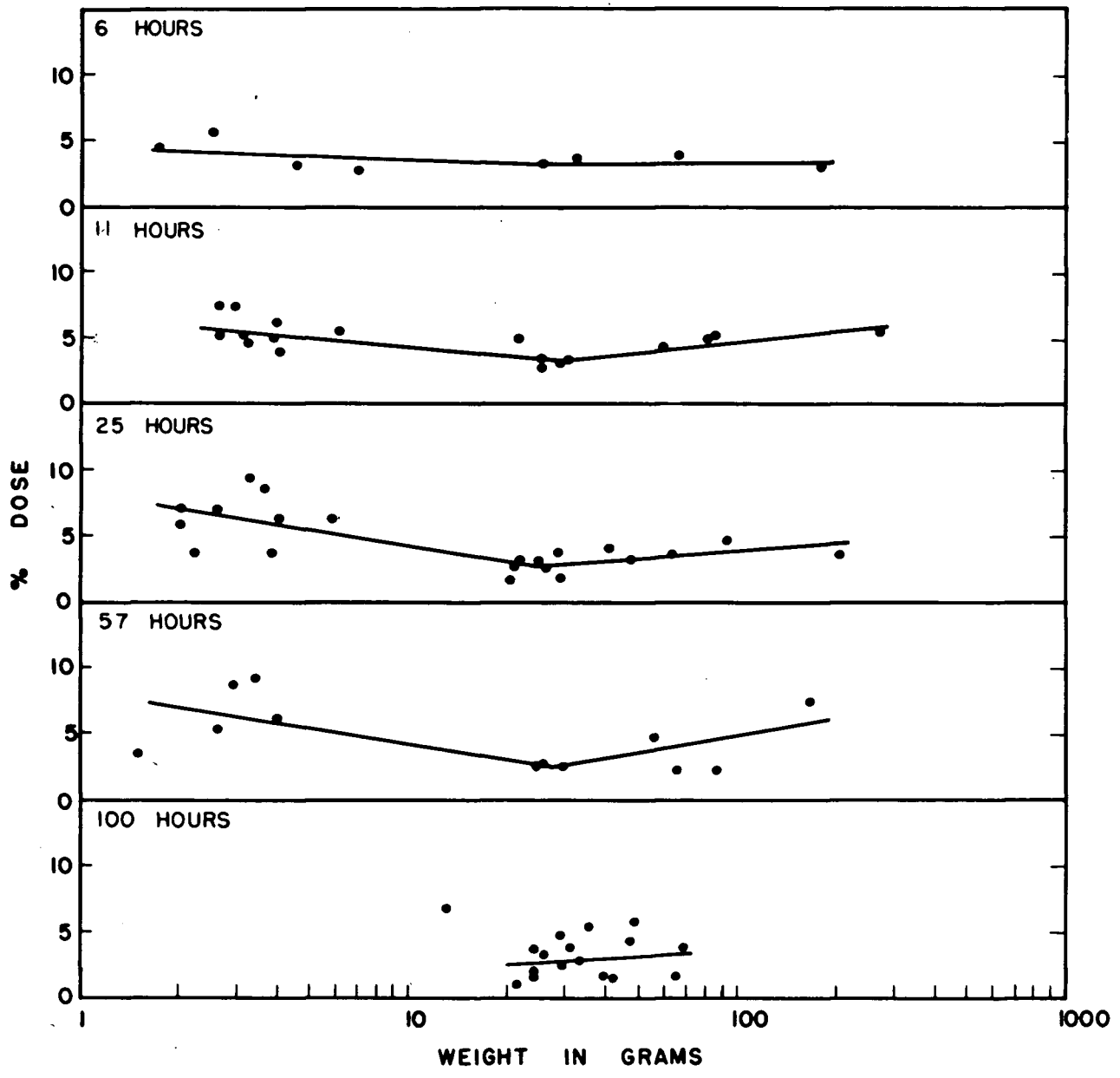

6 HOURS
11 HOURS
25 HOURS
57 HOURS
100 HOURS
% DOSE
0
5
10
1
10
100
1000
WEIGHT IN GRAMS

Figure 26 and 27. Measurements were carried out with sand dab under experimental conditions identical to those used with the flounder (salinity 25°/oo, temperature 15°C.). Thyroid uptake increases proportional to body size, apparently over the entire size range studied (4 to 28 grams). It was not possible to obtain sand dab smaller than about 3 grams and it appears probably that the true picture of the relationship between size and thyroid activity is incomplete in this species for this reason. It will be noted from Figure 26 that there is a suggestion of a diphasic curve at the 24 hour sampling period, indicating that thyroid activity may be greater in individuals smaller than about 4 grams. As already mentioned, the speckled sand dab is a small species rarely exceeding 50 grams in weight and probably reaching maturity at 20 grams. It is therefore not justifiable to compare directly thyroid uptakes of a flounder and a sand dab of the same body weight because the physiological age of the two individuals is entirely different. A 30 gram sand dab is a mature adult, whereas a 30 gram flounder is an immature juvenile. The increase in thyroid activity with body size of flounder above 25 grams implicates the beginning of some reproductive function of the thyroid hormone associated with gonad maturation. Because of the early maturity and small body size of sand dab, gonad maturation probably begins in very small individuals, possibly in 3 or 4 gram fish, as suggested by the 24 hour sampling period (Fig. 26). If so, a 4 gram sand dab may be the physiological age of a 25 gram flounder. Although this suggestion is more in the nature of speculation than deduction, it does offer a possible explanation to what would otherwise be a puzzling contradiction between the effect of body size on thyroid activity of flounder and of sand dab.

Figure 26. Effect of body size on thyroid activity of Citharichthys stigmaeus. Times indicate hours after injection of a tracer dose of I^{131}. Lines fitted by eye. Experimental temperature 15°C., salinity 25 °/oo.

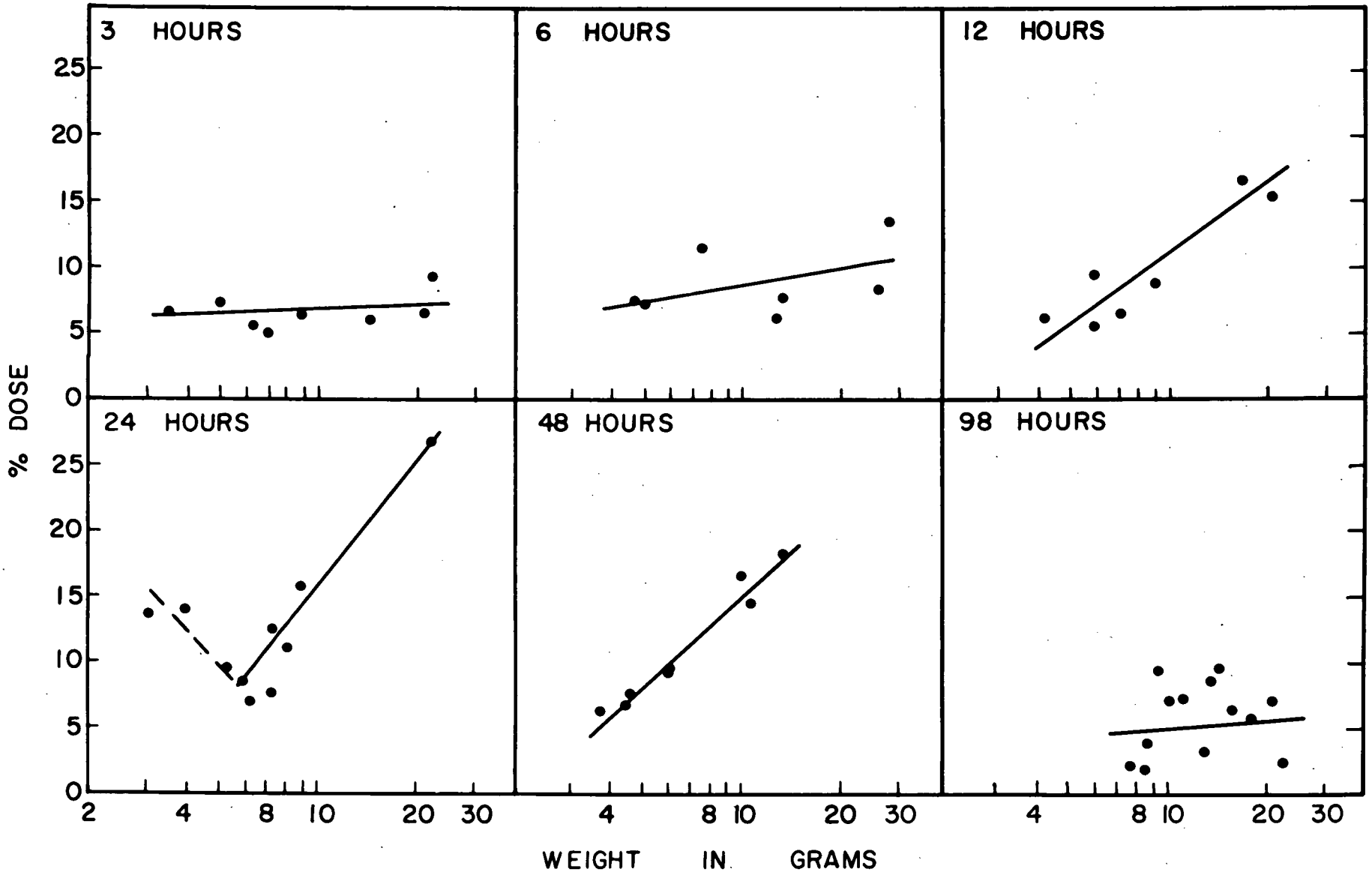
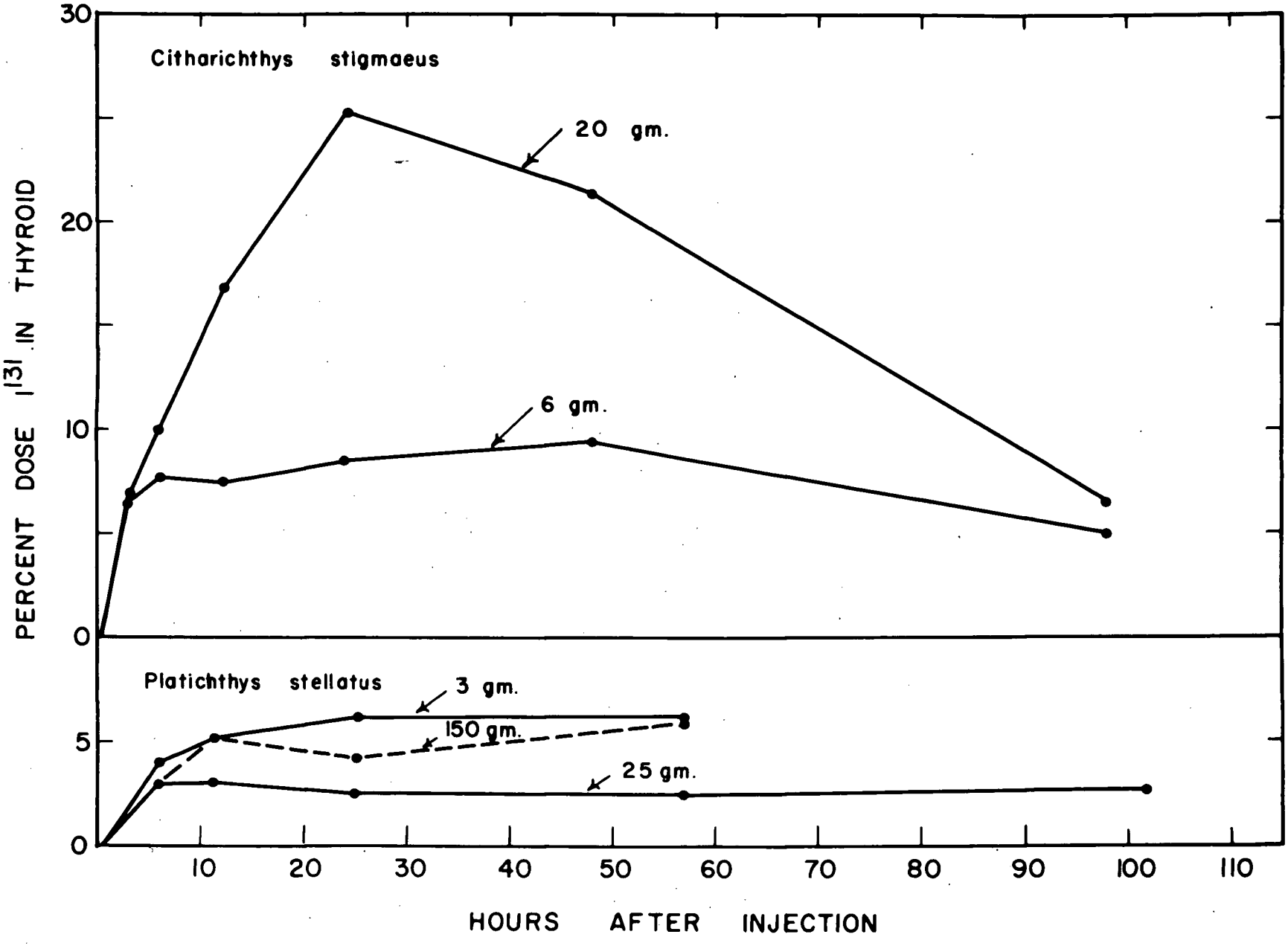

3 HOURS
6 HOURS
12 HOURS
24 HOURS
48 HOURS
98 HOURS
% DOSE
0
5
10
15
20
25
2
4
8
10
20
30
WEIGHT IN GRAMS

Figure 27. Effect of body size on thyroid activity of Citharichthys stigmaeus and Platichthys stellatus. Curves representing fish of the indicated body weight were graphically derived from Figures 25 and 26.


Citharichthys stigmaeus
20 gm.
6 gm.
Platichthys stellatus
3 gm.
150 gm.
25 gm.
PERCENT DOSE I^{131} IN THYROID
HOURS AFTER INJECTION
30
20
10
0
5
0
10
20
30
40
50
60
70
80
90
100
110

VI

DISCUSSION

This investigation of the osmoregulatory metabolism of the starry flounder demonstrates two significant facts and suggests an interdependence between them. The first point is that energy demands are greater in more saline aquatic environments. The second is that the thyroid gland is more active in flounder living in marine habitats. If these factors are interrelated it argues strongly for a calorigenic action of thyroid hormone in at least one species of fish - a fact not yet generally recognized. These points will be discussed in turn. Some ecological implications will finally be considered.

The theoretical energy requirements for osmoregulation in fresh and salt water have already been discussed (Section IV C). It was pointed out that in fresh and salt water of equal though opposite osmotic gradient with respect to the flounders body fluids, the hydrating and dehydrating effects are essentially equal. Fresh water fish excrete excess osmotic water in a dilute and copious urine and regain lost salts in food and by means of ion-absorbing cells in the gills. Marine fish, however, employ a relatively complex osmoregulatory mechanism. To replace water lost osmotically they must drink sea water. Water and univalent ions are absorbed, the salts transported to the gills and actively secreted to the external environment. Divalent ions are removed with the intestinal residue and in the urine with further loss of body water. This method of regaining water lost osmotically to the environment by drinking hypertonic sea water entails considerable "handling" of salts by the organs of exchange and, hence, much active ion transport. Reference was made to the recent research of Ussing (1958) who has been able to quantitate oxygen demands for active cation transport in frog skin. Ussing showed that these energy demands were determined wholly by the amount of ions transported rather than the osmotic gradient present.

If this quantitative relationship exists for all biological membranes, it follows that oxygen demands are greater in sea water because of the relatively circuitous osmoregulatory mechanisms employed by marine fish. Precise measurements of standard metabolic rate in different salinities have demonstrated that flounder consume more oxygen in sea water and thus strongly support the theory of greater energy demands in this medium.

Quantitative differences in active ion transport would appear to be the origin of greater demands for thyroid hormone by marine flounder. A possible point of action of thyroid hormone would be a direct effect on the cells doing osmotic work. However, the question of a calorigenic action of thyroid hormone on the cellular metabolism of fishes is by no means clear. The many attempts made to stimulate oxygen consumption of fish with thyroxine or thyroid treatment and the conflicting results obtained are discussed in several reviews (e.g. Hoar, 1951, 1957; Pickford and Atz, 1957). Using both fresh water and marine fishes, most authors have reported negative results (Drexler and Issekutz, 1935; Root and Etkin, 1937; Etkin, Root and Kofshin, 1940; Hasler and Meyer, 1942; Smith and Everett, 1943; Punt and Jongbloed, 1945; Baraduc, 1954; Hoar, 1958). Chavin and Rossmoore (1956) also found thyroxine to have no effect on goldfish respiration, but obtained significant increases in oxygen consumption with thyrotropin treatment. However, Pickford (Pickford and Atz, 1957) points out that the increase may have been due to contaminating gonadotropin rather than to a direct thyroid stimulation. In spite of these negative reports, a well controlled experiment carried out on goldfish by Muller (1953) showed highly significant increases in oxygen consumption after single injections of thyroxine. Haarmann (1936) found that an optimum dose of thyroxine stimulated the respiration of isolated muscle of carp.

Studies of the effects of thyroidectomy and thyroid inhibition also have given conflicting results. Neither surgical removal nor radiological

destruction of the thyroids of parrot fish (Matty, 1957) and rainbow trout (Fromm and Reineke, 1956) affected their respiration. The use of thyroid chemical inhibitors has frequently yielded negative results (Matthews and Smith, 1947; Chavin and Rossmoore, 1956) although some authors have reported a depression in respiratory rate with the use of these drugs (Osborn, 1951; Zaks and Zamkova, 1952; Muller, 1953). Hoar (1957) has urged that these results be interpreted with caution because of the strong collateral anti-oxidant effect of some antithyroid materials.

It is questionable whether feeding thyroid or immersion in thyroxine is the most effective way to study the calorigenic action of thyroid hormone. These classical procedures are certainly effective in producing all manner of other morphogenetic and metabolic effects in fishes (Hoar, 1957). However it is possible that thyroxine is not the active derivative participating in oxidative metabolism. While the iodinated precursors to thyroxine appear to be the same in all vertebrates (Gorbman, Lissitsky, Micheal and Roche, 1952; Berg and Gorbman, 1953; Gorbman, and Berg, 1955) there is evidence that in mammals thyroxine must be converted to triiodothyronine before it acquires hormonal activity (Barker, 1955; Gross, 1955). Another criticism of the use of administered thyroid compounds or antithyroid drugs is that selection of optimum dosage borders on pure guesswork. In the present study, a correlative decrease in thyroid activity and in metabolic rate has been demonstrated in flounder exposed to a normal physiological situation - a decrease in environmental salinity. This appears to be the first positive demonstration of a calorigenic action of the thyroid hormone in fish not using administered materials such as thyroid hormone or antithyroid compounds. At this point, reference could be made to the work of Olivereau and Francotte-Henry (1956) who have suggested that low metabolic rate and slow growth of African blind cavefish (Caecobarbus geertsi) may be correlated with the inactive thyroid of this animal.

There is yet another deficiency in the experimental procedure of many reported experiments. Frequently inadequate attention has been paid to the importance of the method used in determining oxygen consumption of fish. Fish respiration as measured by the usual method of placing the animal in a closed container and recording the amount of dissolved oxygen consumed per unit of time is notoriously variable. However, it has not been always recognized that much of this variability can be eliminated by measuring standard metabolism following the procedures developed by Fry and his students (Fry, 1957). Changes in respiratory intensity effected by treatment such as feeding thyroxine or dessicated thyroid may be very small and could easily be overshadowed by individual variability unless the precautions outlined by Fry are followed. In this investigation the changes in standard metabolic rate resulting from salinity alterations were not of a large magnitude. The differences could easily be lost in individual variability with a less sensitive method. A case in point is the recent work of Matty (1957) who was unable to demonstrate any change in oxygen consumption of parrot fish (Pseudoscarus guacamaia) after surgical removal of the encapsulated thyroid gland. Matty used the constant-flow principle for his oxygen consumption measurements and was careful to delay sampling for 18 hours to remove the effect of heightened respiration due to handling. However, four deficiencies in procedure may be recognized. First the sample size of 3 fish was far too small. Second, only large fish of 3.0 to 3.2 kg. were used. Third, no effort was made to measure a possible diurnal respiration rhythm, hence, there is no assurance that the measurements represent standard metabolic rate. Finally, the respirometers may have been too small (8.5 times the volume of fish instead of the minimal 1:10 ratio suggested by Geyer and Mann, 1939).

The importance of body size in metabolism experiments is frequently overlooked. Reference to Figure 11b shows that the salinity treatments were inadequate to effect an obvious difference in metabolic rate among large flounder.

Only by inclusion of a large size range of flounder did differences effected by salinity become statistically apparent. Thus, attention to body size is important not only because metabolic rate is weight dependent but also because changes produced by experimental treatment may show up only among small animals.

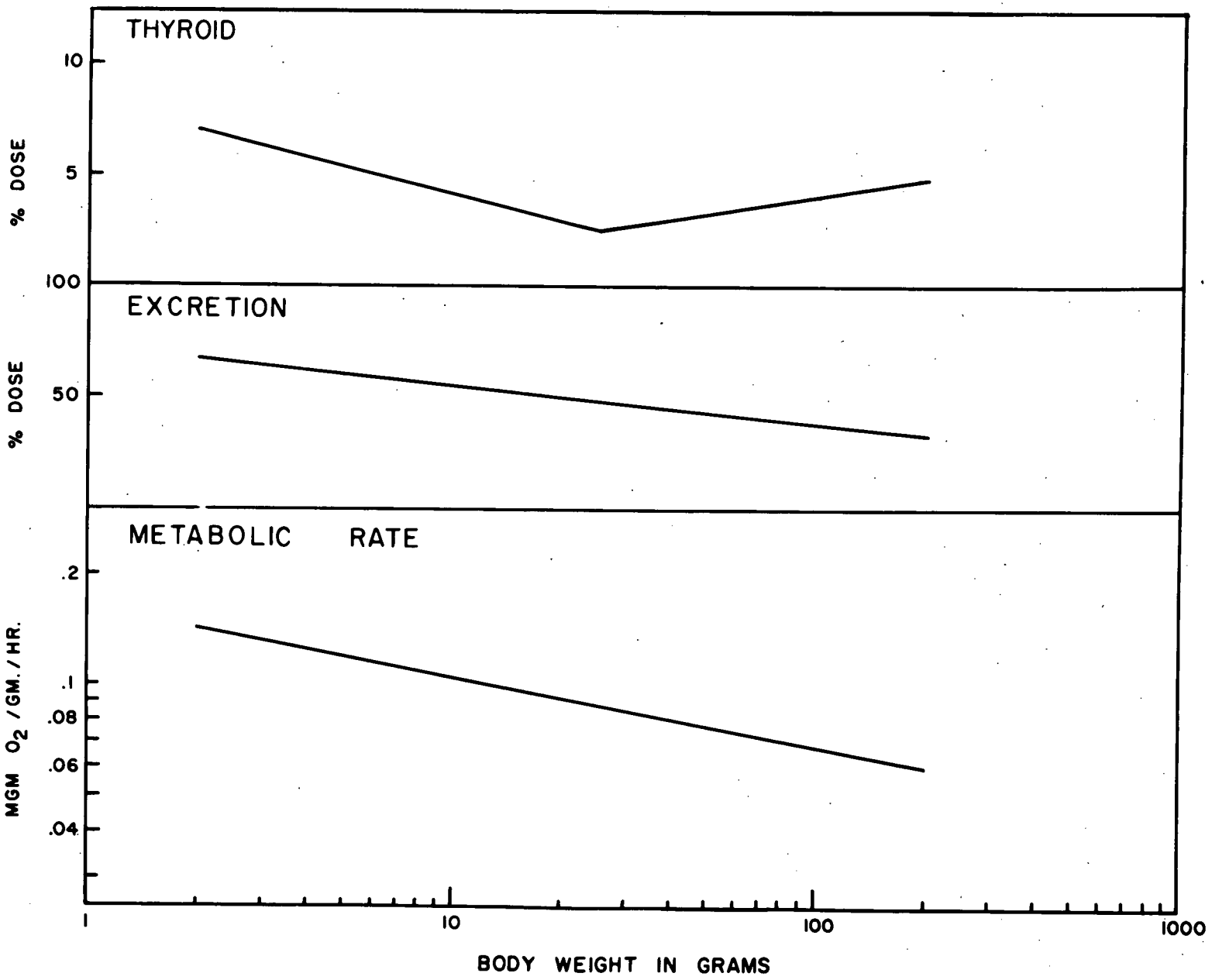
For the reasons discussed above it would appear that the many fruitless attempts to show a calorigenic action of the fish thyroid hormone should by no means be regarded as conclusive evidence that such an action is not present. For one thing it is difficult to see how thyroid hormone can have such apparent effects on protein and carbohydrate metabolism of fishes without influencing in any way oxidative metabolism. Moreover, the well controlled experiment of Muller,, (1953) has demonstrated that thyroid hormone can stimulate oxygen consumption of fish. In view of these facts, the writer would agree with Pickford (Pickford and Atz, 1957) that "it becomes increasingly difficult to deny that thyroid hormone plays some role in the respiration of fishes".

It is usually stated that the primary action of thyroid hormone is its effect on energy metabolism and that the many other effects are secondary results of this primary function. Comparative physiologists, however, tend to dissociate metabolic and morphogenetic effects of thyroid hormone and often argue that the developmental function is quite independent of the calorigenic function (Fleischmann, 1947; Hoar, 1957; and earlier references cited therein). This question cannot be resolved at the present time but several observations made during the course of this study on metabolic rate and thyroid activity of flounder are apropos. As with essentially all animals, metabolic rate of small flounder was greater than large flounder. As a result of their more intense metabolism, smaller flounder excrete a tracer dose of radioiodine considerably faster than do larger individuals (Fig. 21a and 21b). Also disturbances in the osmoconcentration of the body fluids resulting from abrupt salinity alterations were more rapid in smaller flounder (Fig. 3). This is partially due to a more rapid turn-

over of electrolytes and water by the organs of exchange with the environment and partially due to the proportionately greater surface area of small flounder exposed for osmotic movement of water and loss or gain of salts. Thyroid activity, on the other hand, forms a striking exception to the proportional decrease in metabolic activity of physiological processes with increasing body size, for it was shown (Fig. 25) that thyroidal radioiodine uptakes increased in flounder above 30 grams in weight. These relations are summarized diagrammatically in Figure 28. The decrease in thyroid activity with increasing body size of small flounder correlates with a concomitant decrease in metabolic rate. The relation may be a morphogenetic one with thyroid activity decreasing with the decreasing rate of growth with increasing body size. This would be in agreement with the theory that the growth regulating function of the thyroid is more or less independent of the calorigenic function. The effect of thyroid hormone in growth and development is well documented (see reviews by Lynn and Wachowski, 1951; Hoar, 1957 and Pickford and Atz, 1957) and there is general agreement that thyroid activity is high during periods of metamorphosis. Hoar (1951) examined histologically the thyroids of starry flounder in various stages of development. Thyroids appeared active in metamorphosing flounder, while in fully metamorphosed individuals the thyroids had undergone involution. Hoar's findings corresponded with those of Sklower (1939) on the European flounder Pleuronectes platessa. These histological observations strongly indicate that the decrease in thyroid activity of starry flounder with increasing body size is associated with declining rate of growth. It is not clear to what extent thyroid hormone is involved in growth changes, although it is generally felt that it plays a secondary role to the growth hormone but is necessary for the normal expression of the latter.

It was suggested earlier that the systematic increase in thyroid activity in flounder above 30 grams was associated with gonad maturation. Several

Figure 28. Diagrammatic representation of the weight dependency and interrelationships of thyroid activity (% uptake of I^{131}), excretion (% dose of I^{131} excreted 25 hours after injection) and metabolic rate of starry flounder.


THYROID
10
5
% DOSE
100
EXCRETION
% DOSE
50
METABOLIC RATE
.2
.1
.08
.06
.04
MGM O_2 / GM. / HR.
1
10
100
1000
BODY WEIGHT IN GRAMS

investigators have reported that thyroid hormone is necessary for gonad maturation. Thyroid inhibition retards gonad development (Goldsmith et.al., 1943; Barrington and Matty, 1952; Hopper, 1952; Smith, Sladek and Kellner, 1953) while thyroid or thyroxine treatment stimulates the development of secondary sexual characters (Gaiser, 1952; Hopper, 1952). Increased thyroid activity prior or during spawning is well known (Olivereau, 1948, 1949, 1954; Buchmann, 1940; Barrington and Matty, 1954). Hoar, (1951) reported that thyroids of adult starry flounder appeared histologically as active as those metamorphosing flounder. These findings and others are suggestive of some correlate between thyroid activity and the sexual cycle although its significance is presently obscure.

The point of particular interest to be noted from Figure 28 is that the increase in thyroid activity in flounder above 30 grams is without effect on total metabolic rate. If the increase in thyroid radioiodine uptake is truly indicative of greater hormone secretion into the blood stream, it is significant that oxygen consumption is not stimulated. This suggests that the thyroid hormone may have rather specific effects on cells of the body. The increased thyroid activity in sea water is also suggestive of hormone specificity, in this case a direct effect on oxidative metabolism of cells doing osmotic work.

The work of Barker (Barker, 1951; Barker and Schwartz, 1953) indicates that mammalian tissues respond very selectively to thyroid hormone with respect to metabolic rate: liver, kidney, gastric mucosa, salivary glands, pancreas and various muscle tissues responded to thyroid hyper- or hypo-activity, whereas brain, spleen, thymus and various reproductive organs did not respond. Unfortunately, few studies have been carried out on the effect of thyroid hormone on the metabolism of tissues of cold-blooded vertebrates. In the present studies the inflection of the thyroid activity curve in 20-30 gram flounder suggests some direct or indirect involvement between thyroid hormone and gonadal development with no effect on the oxidative metabolism of these tissues. If subsequent research on

the thyroid-reproductive relationship in fishes supports this explanation, the results, together with the suggestion of a specific effect on energy metabolism of osmoregulatory tissues, appear to support the thesis that in lower vertebrates, the developmental and calorigenic actions of thyroid hormone are two independent effects.

The tendency for starry flounder to move into fresh water has certain ecological implications that will now be considered. Because standard metabolic rate of starry flounder is less in fresh water than in sea water, it is easier, at least in this respect, for this species to live in the fresh water environment. The movement of flounder several miles up the Fraser River into entirely fresh water may represent adaptive radiation of this species into a relatively unexploited environment where energy demands are less. It is generally conceded that food is more abundant in the sea than in inland waters. However, the demand for available food is much greater in the sea. Particularly in the coastal area of British Columbia adjacent to rivers, the starry flounder is undoubtedly the most abundant resident species. Intraspecific competition and interspecific competition with the other species such as the common euryhaline armoured sculpin, Leptocottus armatus must be very keen. Movement into fresh water would offer a certain relief from food competition not only with respect to food availability but also because less food must be consumed to meet the lower energy demands for osmotic regulation in fresh water.

However, whereas movement into fresh water is simple and perhaps advantageous, the permanent establishment of a fresh water race of starry flounder is a much greater problem. Starry flounder are marine pelagic spawners as are all the Family Pleuronectidae. Any eggs shed in fresh water would be swept downstream by the current or, if in a more placid situation, sink to the bottom. Even if some larvae succeeded in hatching, it is unlikely that any would survive in an environment to which, as larvae, they are morphologically and physiolo-

gically unsuited. It is evident that there must be drastic changes in mode of reproduction before there can occur the establishment of a residing race of fresh water flounder.

Another point for consideration is the variable ability of the thyroid gland of fish to trap iodine in fresh water. There is evidence that considerable variation exists among fishes in the relative efficiency of the "iodide trap". The alewife (Pomolobus pseudoharengus) and the smelt (Osmerus mordax) are both capable of osmotically regulating in fresh water as well as in their normal marine habitat (Hoar, 1952). However, the thyroids of landlocked alewives were found to be extremely hyper-plastic and showed signs of total exhaustion. At the time of spawning when demands for thyroid hormone are increased, there occurs a spectacular annual mortality of this species. Smelt, on the other hand, have active (although not extremely hyper-plastic) glands in fresh water but experience no mortality at spawning. Because of the very low iodine levels in the Great Lakes region where these fish were taken, the extreme hyper-plasia of the alewife thyroid is probably a goitrogenic reaction to iodine deficiency. Thus, there appears to be a difference in the ability of these two species to trap iodine in fresh water. It is indicated that the development of an efficient iodide trap is an important prerequisite to invasion of fresh water.

The development of euryhalinity and penetration of brackish water by marine fishes is not uncommon. Gunter (1942), after a thorough study of the matter, reports that there are nine times as many marine fishes invading low salinities as fresh water fishes penetrating brackish or marine waters in Middle and North America. He found proportionately more euryhalinity among the phylogenetically primitive orders of fishes. Until recently the theory that the vertebrates originated in fresh water as developed by Romer and Grove (1935) has been generally accepted. Denison (1956) and Robertson (1957), however, present evidence to show that many of the arguments used to support the theory of fresh

water origin are either erroneous or improbable and that a marine origin theory is more solidly supported by existing evidence. If the views of Denison and Robertson are accepted, the development of euryhalinity among the early marine fishes must have preceded freshwater invasion. Perhaps the movement of starry flounder into fresh water exemplifies some of the events which must have occurred eons ago when primitive fishes first developed fresh water tolerance and began invasion of this new habitat.

VII

SUMMARY AND CONCLUSIONS

1. Energy demands for osmotic regulation and the possible osmoregulatory role of the thyroid gland were investigated in the euryhaline starry flounder, Platichthys stellatus.

2. Using a melting-point technique to measure changes in serum osmolarity, it was established that starry flounder possessed efficient osmoregulatory mechanisms in salinities between 0°/oo (fresh water) and 45°/oo (concentrated sea water).

3. After abrupt salinity changes, small flounder experienced more rapid disturbances of serum osmolarity than large flounder. This is related at least in part to the relatively greater surface area of small flounder exposed to osmotic exchange.

4. Standard metabolic rate of starry flounder decreased after transfer to fresh water from sea water. The decrease became more evident with increased time of adaptation in fresh water. Transfer from normal sea water to concentrated sea water resulted in increased metabolic rate of starry flounder and speckled sand dab (Citharichthys stigmaeus). These findings support the theory that energy demands are greater in more saline waters because of the comparative complexity of regulatory mechanisms employed by marine fish.

5. Using the thyroidal uptake of single intraperitoneal doses of radioiodine as a measure of thyroid activity, I^{131} uptake was found to be greater in fresh water adapted flounder. However, reinforcement of iodine deficient fresh water with elemental iodine in amounts equivalent to that present in sea water reduced thyroid uptake of I^{131} to approximately the same as the uptake of sea water flounder.

6. Absorption of I^{131} from the body cavity and blood disappearance rate were more rapid in marine than in fresh water flounder.

7. The decrease in thyroid activity of small flounder with increasing body size appears to be associated with decreasing rate of growth and metabolic rate.

8. Flounder larger than about 30 grams show thyroid activity increasing with body size. This increase in activity has no effect on metabolic rate and may possibly be correlative with gonad maturation in larger flounder.

9. Percentage uptake of radioiodine by the thyroid was shown to be an insensitive and inaccurate criterion for evaluating thyroid activity in different salinities because removal rates of radioiodine from the body and blood differed between fresh water and marine flounder. Using thyroid clearance of radioiodine form the blood as a measure of activity salt water flounder were shown to have much greater thyroid clearance rates and, hence, more active thyroid glands than flounder adapted to fresh water.

10. The greater activity of the thyroid of marine flounder correlates with greater oxygen demands in sea water and indicates a direct or adjunctive osmoregulatory role of the thyroid gland of fish.

APPENDIX

Table I

Analysis of covariance of differences between treatments (times) in the metabolic rate of Platichthys stellatus measured over one 24 hour period. Data of Table IV.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Differences between times	0.72001	103	.00699	3.18***
Total	0.87612	110		

*** Significant at the 1% level.

Table II

Analysis of covariance for the effect of starvation on the standard metabolic rate of spring Platichthys stellatus. Data of Table V.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Differences between 4 days fasted and 7 days fasted	0.08210	31	.00264	5.113**
Total	0.9564	32		
Differences between 7 days fasted and 20 days fasted	0.08557	35	.00245	20.6***
Total	0.13592	36		

** Significant at the 5% level.

*** Significant at the 1% level.

Table III

Analysis of covariance for the effect of starvation on the standard metabolic rate of winter Platichthys stellatus. Data of Table VI.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Difference between 2 and 11 days of fasting	0.1757	40	.00439	37.2***
Total	0.3392	41		

*** Significant at the 1% level.

Table IV

Analysis of covariance of the effect of various salinity treatments on the standard metabolic rate of Platichthys stellatus. Data of Table VII.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Differences between 20°/oo and 8°/oo salinity treatments	0.13135	27	.00486	N.S.
Total	0.13218	28		
Differences between 20°/oo and 0°/oo (20 hour adaptation) salinity treatments	0.10826	27	.00401	7.45**
Total	0.13832	28		
Adaptation time in fresh water (20 hour vs. 4 day adaptation)	0.02945	23	.00128	29.15***
Total	0.06675	24		

** Significant at the 5% level.

*** Significant at the 1% level.

Table V

Analysis of covariance of effect of increased salinity on the standard metabolic rate of winter Platichthys stellatus. Data of Table VIII.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Differences between 25°/oo and 49°/oo salinity treatments	0.16592	38	.00437	55.1***
Total	0.40647	39		

*** Significant at the 1% level.

Table VI

Analysis of covariance of the effect of various salinity treatments on the standard metabolic rate of Platichthys stellatus. Data of Table IX.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Differences between 22.8°/oo and 0°/oo (20 hour adaptation) salinity treatments	0.07219	31	.00232	3.04*
Total	0.07925	32		
Differences between 26°/oo and 0°/oo (5 day adaptation) salinity treatments	0.12139	42	.00289	11.1***
Total	0.15335	43		
Differences between 25°/oo and 43.2°/oo salinity treatments	0.13670	42	.00325	17.3***
Total	0.19301	43		

* Significant at the 10% level.

*** Significant at the 1% level.

Table VII

Analysis of covariance of the effect of increased salinity on the standard metabolic rate of Citharichthys stigmaeus. Data of Table X.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio
Difference between 24.4°/oo and 43.4°/oo salinity treatment	0.11929	30	.00397	44.6***
Total	0.29649	31		

*** Significant at the 1% level.

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