# A STUDY OF THYROID FUNCTION IN FUNDULUS HETEROCLITUS [1, 2]

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The role of the thyroid gland in regulating the metabolic rate, and its effect on growth and other physiological functions in higher vertebrates have been well established, but in fish, despite the many studies using anti-thyroid drugs, thyroxine, thyroid extract, and thyrotropin, its function is still obscure. The diffuse nature of the thyroid gland of most teleost fishes has rendered the study of its function extremely difficult; surgical removal is impossible except in a very few species where the gland is encapsulated, and the use of anti-thyroid drugs introduces disadvantageous collateral effects (reviewed by Chambers, 1953). The availability of radioactive iodine has now made possible a new technique for the extirpation of the thyroid gland, especially suited to one of diffuse nature as found in these fish. La Roche and Leblond (1954) were the first to investigate the problem of radiation thyroidectomy in fishes. They found that total destruction of the thyroid in salmon (*Salmo salar* L.) required repeated injections of large, but progressively downgraded doses of $I^{131}$. Fish weighing 30–32 grams at the start of the experiment received 100, 50, 40 and 30 $\mu$C at the rate of one dose per month. Arvy, Fontaine and Gabe (1956) also used a series of injections, but only claimed to have achieved a state of hypothyroidism in rainbow trout (*Salmo gairdneri* Richardson). They gave a total dose of 260 $\mu$C in three injections at 30-day intervals, to fish weighing about fifty grams. More recently, Fromm and Reineke (1957) have reported successful destruction of the thyroid after a single injection of 250 $\mu$C to fingerling trout weighing 3.8–6 grams. In an abstract, which has not been reported in detail, Baker, Berg, Gorbman and Gordon (1955) described the effects of partial or complete thyroid destruction in platyfish by the addition of 4.5–7 $\mu$C of $I^{131}$ to 200 ml. of aquarium water. The period of exposure was 24–48 hours, but more complete destruction resulted from the longer treatment. Fontaine, de la Querrière and Raffy (1957), in a study of the respiratory metabolism of hypophysectomized eels, noted a fall in the oxygen consumption 48 hours after the injection of 334 $\mu$C of $I^{131}$ into fish weighing about 70 grams. This was followed by a gradual return towards normal over a period of several weeks. Olivereau (1957) has discussed the problem of dosage, tissue damage, and regeneration of the thyroid in eels treated with radioactive iodine.

In this study *Fundulus heteroclitus* was chosen as an experimental fish, not

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only for reasons of its hardiness and availability, but since it is a euryhaline species, it afforded the opportunity to study the possible role of the thyroid in water and salt regulation. Following an initial pilot experiment to determine the dosage of $I^{131}$ necessary for thyroidectomy, the following experiments were designed to study the physiological effects of thyroid deficiency on growth and ability to osmoregulate, as well as effects on other organs and tissues that might possibly be dependent on thyroid function.

## Materials and Methods

The fish used were *Fundulus heteroclitus* males of about 7.5 cm. in length, caught in the New Haven area in August, 1956. They were placed in storage tanks kept at 20° C. for two months before use, at which time they were measured, numbered, and selected for uniformity of weight of approximately five grams. The results of the previous experiment to determine dosage indicated that dosage levels somewhat lower than 10 $\mu$C/gm. wt. spaced over a period of several months would be the most effective procedure for destroying the active as well as the initially inactive follicles. Accordingly, in this experiment a series of graded doses was given, 25, 15, and 10 $\mu$C, spaced about five weeks apart. Thus, with five-gram fish, the doses were approximately 5, 3, and 2 $\mu$C per gram weight of fish.

On October 23, the thirty-six fish selected for $I^{131}$ treatment were given a dose of 5 $\mu$g per fish of thyrotropin (Armour 317–51) ; on the following day they were given the first injection of $I^{131}$, 25 $\mu$C per fish in a volume of .05 ml. The injected fish were placed in five-gallon wide-mouth carboys provided with aeration and means for feeding and changing water without handling the fish, and after eight days they were returned to their regular aquaria. On November 27 the second dose of iodine was given, 15 $\mu$C per fish, with an injection of 5 $\mu$g of thyrotropin given two days before. The third iodine injection of 10 $\mu$C per fish was given on January 4, 1957, following the usual dose of thyrotropin. These fish were subsequently screened by the tracer method described below, and those showing the greatest impairment of thyroid activity were set aside for further experiments. The fish were anaesthetized with tricaine methane sulfonate (MS 222) for injections and later screening operations.

The fish were fed once daily with Aronson's formula fish food, consisting of a cooked mixture of ground beef liver, kidney, greens, dried shrimp, and Pablum. The temperature in all tanks was kept at 20° C. and illumination was ten hours per day.

### *Experimental setup for osmoregulation studies*

To study the physiological effects of hypothyroidism following direct transfer of fish from sea water to fresh water, it is necessary to maintain tanks with sea water of constant salinity as well as several with running fresh water. A small circulating sea water system of simple design was used, with a 55-gallon polyethylene reservoir drum feeding by gravity into the aquaria, the overflow draining through a filter, and subsequently returned to the reservoir by means of a hard rubber pump. The salinity of this system was kept at 26 ‰, with a pH of 7.5 and oxygen content of 4.28 ml./liter.

A dechlorinating system for tap water similar to that described by Burden (1956) was the fresh-water source. The pH was 6.9, oxygen content 5.46 ml./liter, and a flow rate of 350–400 ml. per minute was maintained in each of the tanks.

*Autopsy procedures*

All fish which died during the iodine treatment or prior to the osmoregulation experiment were autopsied to determine the possible causes of death, and the thyroids were fixed in Bouin's for histological examination. At the termination of the osmoregulation experiment, the remaining fish were autopsied, measurements of standard length, weight, and testis weight were made, the thyroid was fixed in Bouin's, and blood samples were taken for hematology and chloride titration, as described below.

*Hematology*

Red and white cell counts, thrombocytes, and per cent hemoglobin were determined on blood samples which were allowed to drop on siliconized slides from the cut end of the tail. Heparin was used on the razor blade. The procedures will be described elsewhere by Dr. Anne M. Slicher, who made this study.

*Blood chloride*

Blood chloride determinations were made using a micro-adaptation of the method of Schales and Schales (see Hawk, Oser and Summerson, 1954). Whole blood was collected from tail cuts, centrifuged in an air-driven "spinning top" rotor, and 4.8-$\mu$l samples of serum were pipetted into titration vessels, closed with paraffined corks and frozen. Before titration the samples were diluted with 42.6 $\mu$l of distilled water to which indicator had been added (3 ml. stock indicator/50 ml. solution). It was found necessary to add about six drops of 2.0 N $HNO_3$ to the 50 ml. of diluted indicator in order to release the bound chloride in the sample.

To check the accuracy of the method, standard human serum (Hyland Laboratories, Los Angeles, lot 369E6) with known NaCl was titrated, using the same amounts and procedures as with the fish preparation. The standard contained 544 mg% with an acceptable range of 533–555 mg%. The test titrations gave a value of 549 mg%, well within the range.

*Screening method for determining degree of thyroidectomy*

Although all fish were ultimately examined histologically to determine the degree of thyroidectomy, it was desirable to know how effectively the thyroid had been destroyed before using the fish for experimental purposes. Thus a method using a tracer dose of $I^{131}$ to measure the rate of activity loss in the throat region was worked out and a device was designed to hold the fish over a Geiger counter so that comparison readings could be made between normal and treated fish.

A lead block was cast around the mold of a fish to serve as shielding, as well as a holder for the experimental fish. A hole was drilled through the bottom of the block to connect with the thyroid region of the fish, and the block was mounted on a wood frame so that the opening coincided with the ¼-inch end window of a Geiger-Müller tube (Mark 1, Model 105. Radiation Counter Laboratories, Inc.,

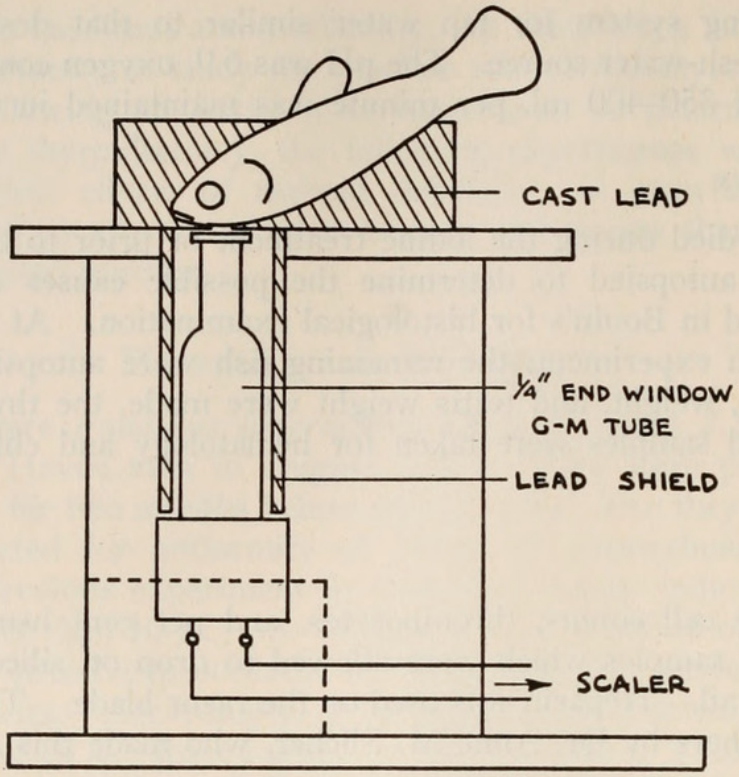


FIGURE 1. Counter arrangement for measuring activity of thyroid area.

Skokie, Ill.). Lead shielding was found to be necessary around the tube, and its photosensitivity was obviated by cementing a thin plastic film over the opening in the bottom of the block and coating with "Aquadag" (see Fig. 1).

A series of trial injections of $I^{131}$ indicated that a dose of 2.5 $\mu$C per five-gram fish gave an adequate counting rate, and since this was also in line with tracer doses used by Gorbman and Berg (1955) and others, it was subsequently used in this screening procedure.

## RESULTS

### *Histological studies of the thyroid*

Following fixation in Bouin's solution at the time of autopsy, the thyroid regions of the experimental and control fish were examined histologically to determine the degree of thyroidectomy. Serial sections were made through the entire region, and an evaluation was made based on the relative number of follicles present, the height of the epithelial cells, and amount of colloid, in relation to the control fish. Few of the treated fish showed a total lack of follicles, but it was interesting to note that these fish, and others with very few follicles, did not survive to the end of the experiment. There was a great range in the amount of thyroid tissue present. However, while some fish showed evidence of considerable regeneration, in the majority of fish regeneration was present to a much lesser extent, and on the whole they could be considered markedly hypothyroid.

### *Uptake of a tracer dose of $I^{131}$ in hypothyroid and control fish*

The most promising means of evaluating the tracer data appeared to be a simple comparison of rate of activity loss in the thyroid region of the treated (hypothyroid)

fish with that of the controls. In the first of two groups run to test the screening method, the initial readings were made four hours following the injection of $I^{131}$. In the hypothyroid fish, the counting rate ranged from 193 to 398 counts per minute, with a mean of 297 counts per minute. The controls ranged from 231 to 465 counts per minute, with a mean of 345. Since there was already a drop in the activity in the hypothyroid fish, as compared with the controls, a second group of fish was screened in the same manner, but taking the initial reading at one and a half hours, rather than four. Here the range in the hypothyroid fish was 313 to

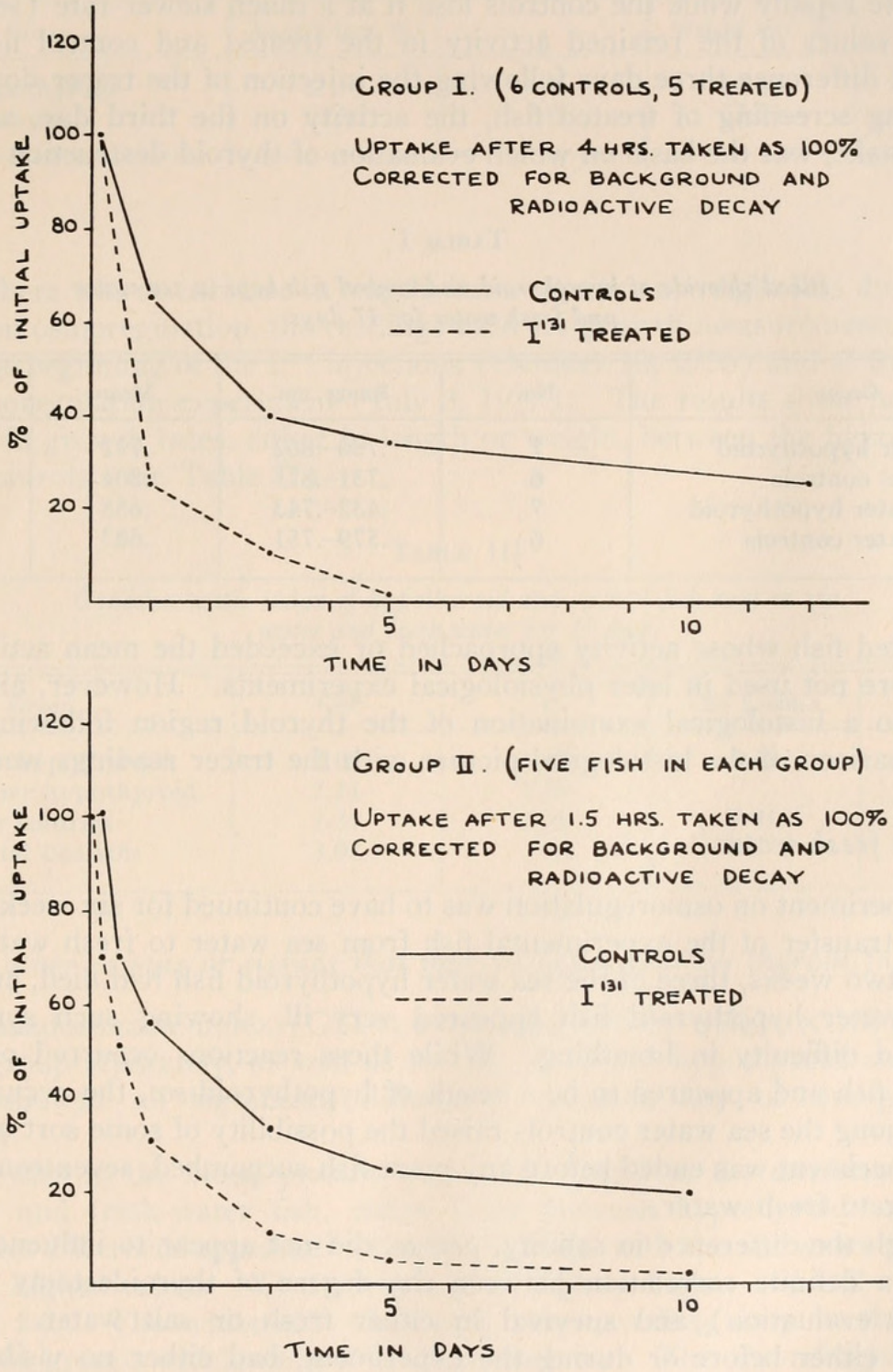


FIGURE 2. Activity loss in control and treated fish following a tracer dose of 2.5 $\mu$C of $I^{131}$.

447 counts per minute, with a mean of 383. The controls ranged from 315 to 507 counts per minute with a mean of 385. However, in both groups the subsequent readings on each fish were converted to percentage of its initial reading, the best way to take into account the variations between individual fish resulting from weight differences, thyroid activity, or unavoidable variations in dose.

While the activity of hypothyroid fish dropped immediately, the activity of the controls continued to increase for several hours before beginning to drop off. The peak appears to be around three to four hours following injection. The two sets of curves show essentially the same pattern: the hypothyroid fish losing activity quite rapidly while the controls lose it at a much slower rate (see Fig. 2). The mean values of the retained activity in the treated and control fish showed the greatest difference three days following the injection of the tracer dose; thus in the following screening of treated fish, the activity on the third day, as per cent of initial uptake, was the basis on which evaluation of thyroid destruction was made.

TABLE I

*Blood chloride of hypothyroid and control fish kept in sea water and fresh water for 17 days*

| Group | No. | Range, gm. | Mean | s |
|---|---|---|---|---|
| Sea water hypothyroid | 7 | .730–.862 | .792 | .045 |
| Sea water controls | 6 | .731–.875 | .804 | .055 |
| Fresh water hypothyroid | 7 | .432–.743 | .655 | .113 |
| Fresh water controls | 6 | .579–.751 | .683 | .068 |

Those treated fish whose activity approached or exceeded the mean activity of the controls were not used in later physiological experiments. However, all fish were subjected to a histological examination of the thyroid region following autopsy, and a comparison of the histological picture with the tracer readings was made.

*Survival in fresh water*

The experiment on osmoregulation was to have continued for six weeks following the abrupt transfer of the experimental fish from sea water to fresh water. However, after two weeks, three of the sea water hypothyroid fish had died, and three of the fresh water hypothyroid fish appeared very ill, showing such symptoms as tremors and difficulty in breathing. While these reactions occurred only among the treated fish and appeared to be a result of hypothyroidism, the occurrence of a sick fish among the sea water controls raised the possibility of some sort of infection, and the experiment was ended before any more fish succumbed, seventeen days after the transfer to fresh water.

Although the difference in salinity, *per se,* did not appear to influence survival, there was a definite correlation between the degree of thyroidectomy (based on histological evaluation) and survival in either fresh or salt water. Seven fish which died either before or during the experiment, had either no visible follicles, or extremely few.

*Blood chloride*

All fish in fresh water showed a definite drop in blood chloride (about 24 per cent), but within each group (*i.e.*, salt and fresh water) there was no significant difference between the hypothyroid and control fish (see Table I).

TABLE II

*Growth of treated and control fish during nine months following the beginning of $I^{131}$ treatment*

| Group | Length incr. % | s | Weight incr. % | s |
|---|---|---|---|---|
| Hypothyroid | 11.71 | 3.9 | 54.66 | 23.1 |
| Controls | 13.02 | 7.0 | 57.74 | 29.9 |

*Growth*

Since there was no increase in length, but a significant weight loss during the experiment on osmoregulation, the readings used for growth measurements were those taken at the beginning of the $I^{131}$ injections (October 13, 1956) and at the beginning of the osmoregulation experiment (July 3, 1957). The results show no significant difference in growth rates, either in length or weight, between the hypothyroid fish and the controls (see Table II).

TABLE III

*Gonadosomatic index of hypothyroid and control fish kept in sea water and fresh water for 17 days*

| Group | GSI | s | GSI (comb.) | s |
|---|---|---|---|---|
| Sea water hypothyroid | 3.09 | 1.57 | 2.47 | 1.62 |
| Fresh water hypothyroid | 2.14 | 1.39 | | |
| Sea water controls | 2.51 | 1.49 | 2.91 | 1.20 |
| Fresh water controls | 3.01 | .69 | | |

*Effect on other organs or tissues that may be dependent on thyroid function*

The gonadosomatic index (GSI = testis weight/body weight × 100), calculated for each group separately, as well as for the combined hypothyroid and combined controls, showed no significant differences between any of the groups (see Table III).

Differences in the blood picture were to be found in the combined groups of sea water and fresh-water fish, rather than between hypothyroid and controls. The greatest difference appeared in the hemoglobin, with the sea water fish showing somewhat higher values. There was also a slightly higher red cell count in the sea water fish. The white cells showed a very puzzling drop in the sea water hypothyroid group, which cannot be accounted for. The thrombocytes show no significant differences (see Table IV).

TABLE IV

*Hemoglobin titers, red cell, white cell and thrombocyte counts of hypothyroid and control fish kept in sea water and fresh water for 17 days*

| Group | Hb% | s | RBC* | s | WBC | s | Thromb. | s |
|---|---|---|---|---|---|---|---|---|
| S.W. hypothyroid | 60 | 11.3 | 4.32 | .69 | 5.54 | .48 | 12.7 | 1.9 |
| F.W. hypothyroid | 55 | 11.4 | 4.11 | .54 | 9.92 | 3.5 | 12.6 | 1.8 |
| S.W. controls | 63 | 7.9 | 4.64 | .83 | 9.21 | 3.5 | 14.0 | 4.0 |
| F.W. controls | 50 | 13.6 | 4.51 | .86 | 9.20 | 1.5 | 14.2 | 2.4 |

* RBC in millions, WBC in 1000's, Thrombocytes in 10,000's.

## DISCUSSION

### *Osmoregulation*

There is good evidence to support the idea that the thyroid gland plays some role in the salinity tolerance of fish. It is well known that many species of fish show an activated thyroid gland during spawning migration from the sea to fresh water; experimental work of Olivereau (1948) and Leloup (1948) on several species of marine teleosts has shown that there is a strong activation of the thyroid gland with decreasing salinity of the medium; Fontaine and Callamand (quoted by Fontaine, 1956) have shown that thyroxine injections increase the survival time of several marine fish when transferred to fresh water. Thus it was of interest to test the salinity tolerance of hypothyroid fish, in terms of survival time and blood chloride concentrations.

Survival time, however, proved to be a function of degree of thyroidectomy, rather than of the salinity of the medium. Almost all of the treated fish which died during the course of the experiment were those with no follicles, or extremely few.

The blood chloride concentrations were obviously a function of the salt concentration of the medium. All fish in fresh water showed a definite drop in blood chloride (about 24 per cent) but there was no significant difference between the hypothyroid fish and the control fish. Burden (1956), on the other hand, found no change in blood chloride concentration of *Fundulus* kept in fresh water for eight days. Since the same method was used for the chloride determinations reported here, Burden's higher results might be attributed to the difficulty in determining the end point when using non-deproteinized serum. Sex and season were the same, and variations attributable to such causes are excluded. However, Burden's experiments were made at a lower temperature (15° C.) and this may have contributed to a slower period of adjustment to the new external environment. It is possible that there is a gradual chloride loss which was not detectable during the short period of time employed by Burden, although V. S. Black (1948) demonstrated a loss of body chloride in *Fundulus heteroclitus* transferred directly from sea water to fresh water, with a stable level of about 60 per cent reached after the fourth day. Bergeron (1956) has shown that *Fundulus* maintains a constant blood osmotic pressure in both salt and fresh water, confirming earlier work of Garrard (1935). It may be that the osmotic pressure is maintained by an exchange of carbonate ions for chloride, since the alkali reserve of the blood of marine fish is lower than that of fresh water species, and there is a relative decrease in the

bicarbonate ion when migrating eels are transferred to sea water (Drilhon and Florence, 1936; Fontaine and Boucher-Firley, 1934). Work of Koch and Heuts (1942) and Heuts (1943) showed that changes in serum osmotic pressure of mature sticklebacks transferred to sea water could not be entirely accounted for by changes in blood chloride. In this light, further experiments seem to be called for to determine the rate of blood chloride loss following transfer from sea water to fresh water, and the factors involved in maintaining osmotic pressure, with special emphasis on the alkali reserve.

*Other effects*

The hemoglobin and red cell counts, like the blood chlorides, indicate a dependence on the medium, with no apparent influence by the presence or absence of thyroid tissue. While the increased values found in the sea water fish may be accounted for by the lower oxygen content of that medium (see Prosser, Barr, Pinc and Lauer, 1957), or by an increased energy demand for fish in higher salinities, as found by Hickman (1958) in *Platichthys stellatus,* the starry flounder, the differences are small and cannot be considered significant.

Other than the effect on survival, where the actual cause of death is unknown, there was no apparent effect of hypothyroidism on any of the physiological processes studied here. Growth rates were not affected by the hypothyroid condition, nor was there any effect on gonadosomatic index.

The literature devoted to thyroid regulation of growth in teleost fishes is confusing. With the species *Lebistes reticulatus* alone, anti-thyroid drugs have been reported to retard growth (Hopper, 1950, 1952; Gaiser, 1952; Vivien and Gaiser, 1952; Smith, Sladek and Kellner, 1953), while Fortune (1955) found no effect on growth in either *Phoxinus* or *Lebistes* (see Pickford and Atz, 1957). Possibly collateral toxic effects of the anti-thyroid drugs may be responsible for the retardation of growth, and it is of interest that in salmon parr thyroidectomized with $I^{131}$ no effect on growth was found (La Roche and Leblond, 1954). Thyrotropin injected into hypophysectomized *Fundulus* was found by Pickford (1954) to have no effect in restoring growth, indicating that the thyroid at least has no direct effect on growth. However, in such fish, as in hypophysectomized rats, thyroid stimulation undoubtedly enhances the response to exogenous growth hormone (Pickford and Atz, 1957, p. 99).

Data concerning the role of the thyroid in sexual maturation are no less conflicting than those on growth. However, studies with anti-thyroid drugs strongly indicate that the thyroid is instrumental in the maturation of the gonads (reviewed by Pickford and Atz, 1957). While Barrington (1954) and Fortune (1955) found that *Phoxinus* could reach sexual maturity despite treatment with thiouracil, it is possible that there was not complete inhibition of thyroid function, as in the work reported here, and that this minimal amount of hormone was sufficient to permit sexual maturation.

*Screening method*

There appears to be a considerable discrepancy between the evaluation obtained with the tracer technique and that from the histological examination, based on

apparent number and size of follicles and cell height. Since there was a time lapse of approximately three months between the time of tracer screening and autopsy, the most plausible explanation for the difference is the regeneration of thyroid tissue in that period. Olivereau (1957), in her work on radiothyroidectomy of eels, found that in fish of about 40 grams that had received a total of 1000 $\mu$C of $I^{131}$ in three doses, functional thyroid tissue had already regenerated after two months, and seven months later she found complete absence of the thyroid in only three out of fifteen fish. It would thus be advisable to repeat the tracer screening just prior to autopsy.

## Summary

Thyroidectomy of *Fundulus heteroclitus* was attempted with the use of radioactive iodine, administered in three doses of 25, 15, and 10 $\mu$C per five-gram fish at intervals of five weeks. A screening method was developed whereby the degree of thyroidectomy could be determined by the rate of activity loss in the throat region of the fish following a tracer dose of $I^{131}$. Thyroidectomy was not complete, and in some cases there was considerable regeneration. However, in general, the resulting condition was one of extreme hypothyroidism, and physiological studies conducted with these fish yielded the following results:

1. There was no special effect on the fishes' ability to survive in fresh water, although there seemed to be an impairment of their ability to survive at all, in either salt or fresh water. Deaths occurring during the experiment in general involved fish with very few follicles or no thyroid tissue remaining.
2. Blood chloride titers were a function of the salinity of the medium and were not affected by lack of thyroid.
3. Hemoglobin titers and red cell counts indicated an effect of the medium, and were not influenced by lack of thyroid.
4. Hypothyroidism had no effect on growth, either in length or weight.
5. Hypothyroidism had no effect on the gonadosomatic index.

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