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THE UNIVERSITY OF CALGARY

ANNUAL VARIATIONS IN CIRCULATING THYROID HORMONES IN THE BROOK TROUT, *Salvelinus fontinalis* (Mitchill).

by

Bruce Alan White

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "ANNUAL VARIATIONS IN CIRCULATING THYROID HORMONES IN THE BROOK TROUT, *Salvelinus fontinalis* (Mitchill)" submitted by BRUCE ALAN WHITE in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Circulating levels of the two thyroid hormones, L-thyroxine (T_4) and 3,5,3'-L-triiodothyronine (T_3) were measured by radioimmunoassay in plasma samples of brook trout, *Salvelinus fontinalis* (Mitchill) exposed to natural daylengths and to an accelerated light regime. The relative affinity of thyroid hormone-binding proteins was assessed in plasma from brook trout and two mammalian species using Sephadex gel column chromatography. An attempt was made to measure 5-methoxy-N-acetyl tryptamine (melatonin) spectrophotofluorometrically in pooled pineal samples of brook and rainbow trout.

Mean circulating concentrations of total T_4 ranged from 0.8 to 3.7 ng/ml and mean circulating total T_3 levels varied from 2.4 to 8.6 ng/ml over a year in fish exposed to natural conditions. Both hormones reach maximum levels in April and minimum levels in early November at the time of spawning. The plasma levels of both hormones exhibited daily fluctuations with lowest values occurring in the early morning. T_3 concentrations were greater than those of T_4 in all but one of 68 fish examined.

The rate of reproductive development in fish exposed to an accelerated photoperiodic regime did not differ from that in trout exposed to natural changes in daylength. These fish had lower levels of T_4 and T_3 compared with the corresponding monthly levels in fish held under natural conditions. The fish in the experimental light regime did not feed well and this probably accounts for the low hormone levels.

Thyroid hormone-protein association was found to be weaker in trout plasma than in mammalian samples. T_4 binding was weaker than that of T_3

in fish while the reverse was found in mammalian samples.

The limit of detection and extraction efficiency of the melatonin assay were shown to be satisfactory when compared to previous studies. However, no melatonin could be detected in any of the pineal samples.

The data clearly demonstrate annual variations of circulating levels of the two thyroid hormones. These levels are compared with published plasma concentration of T_3 and T_4 for other vertebrates. The levels in trout are relatively low, possibly due to loose associations with plasma proteins and low ambiant iodine concentrations.

The possible causal relations between thyroid hormone levels and environmental factors, reproductive development and growth are discussed.

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Introduction

The Thyroid Axis in Teleost Fish

The thyroid gland in most teleosts is not a compact organ. The follicles frequently are scattered over a wide area extending from the lower jaw to the kidney. However, the thyroid follicle of teleosts is similar to that of all other vertebrates with respect to morphological and biochemical characteristics (Gorbman, 1969). In fish, the thyroid and ovaries are the major sites of iodide concentration; yet only the thyroid has the capacity for iodinated thyronine synthesis (Leloup and Fontaine, 1960). Iodide is actively taken up by the follicular epithelial cell, dehalogenated and transported to the apical pole where it is released into the follicular lumen. Peroxidase-mediated oxidation of iodide to iodine and iodination of tyrosine residues within thyroglobulin occur at the microvilli-colloid interface. Coupling within thyroglobulin of iodinated precursors via ether linkage creates the storage forms of the two thyroid hormones, 3,5,3'-L-triiodothyronine (T_3) and L-thyroxine (T_4) (for a complete treatment of thyroid hormone biosynthesis, see Taurog, 1974).

In mammals, both T_3 and T_4 are secreted by the thyroid. Additionally, T_3 is derived from T_4 by deiodination. It is estimated that 32-63% of total body T_3 is derived from peripheral deiodination of T_4 (Oppenheimer and Surks, 1974). T_3 is generally considered to be the more active form (Oppenheimer and Surks, 1975) and T_4 a prohormone of T_3. However, Oppenheimer and Surks, (1974, p.211) emphasize that "...neither the T_3-to-T_4 potency ratio nor the T_4-to-T_3 conversion rate are known with sufficient accuracy to definitively exclude some independent hormonal

activity for T_4". In teleosts, T_3 is detoxified more slowly than T_4 (Higgs, 1976), but the extent of T_4 detoxification and the T_4 to T_3 conversion rate are not known. There is no information in fish to indicate which iodothyronine(s) has intrinsic biological activity.

Evidence from chromatographic analyses exists for only T_4 (*Fundulus heteroclitus*; Berg *et al.*, 1959), only T_3 (*Tinca tinca*; Chavin and Brown man, 1965) or both thyroid hormones (*Oncorhynchus kisutch*; Osborn and Simpson, 1969) being synthesized by the teleost thyroid. However, reservations about conclusions implicit in previous studies arise for two reasons. First, the detection limits of the assays used are insensitive relative to the currently available saturation binding techniques. Second, the authors often reported measurements on single samples of fish; they did not consider effects of varying environmental conditions on thyroid activity. Thyroid hormone production is affected both quantitatively and qualitatively by internal controls (e.g., thyroid hormone status-pituitary-hypothalamus closed-loop servomechanisms), external factors (e.g., iodine concentration of water) or both (e.g., temperature or photoperiod via hypothalamohypophyseal open-loop control) (Hoar, 1959; Berg *et al.*, 1959; Hickman, 1962; Gorbman, 1969).

In mammals, T_4 exists in equilibrium with three thyroid-binding proteins (TBP), thyroxine-binding globulin (TBG), thyroxine-binding prealbumin, and albumin. T_3 is in equilibrium with TBG and, to a small extent albumin (Woeber and Ingbar, 1974). A very low amount of both hormones circulate as free hormone (0.01-0.02% total T_4 and 0.1-0.2% total T_3; van Middlesworth, 1974). Free thyroid hormones are available to intracellular receptors and can readily be supplied by the bound portion during times of increased metabolic demand (Oppenheimer and Surks,

1974). There is no specifically-binding TBG present in teleosts, but only a non-specific association with albumins and prealbumins (Farer *et al.*, 1962; Tanabe *et al.*, 1969; Refetoff *et al.*, 1970).

As in other vertebrates, control of the teleost thyroid involves neural pathways whose activity is integrated in the hypothalamus. From the hypothalamus, signals are sent to the pituitary which modulate the response of hypophyseal thyrotropic cells (TSH cells) to feedback control by thyroid hormones (see Reichlin *et al.*, 1972, for discussion of this concept). Stimulatory pituitary control over the teleost thyroid is well-established (Pickford , 1957; Sage and Bern, 1971). TSH cells are located in the proximal pars distalis of the pituitary (Sage and Bern, 1971) and a thyroid-stimulating hormone (TSH) has been partially purified (Y.A. Fontaine, 1969).

The anterior pituitary of many teleosts is directly innervated by type "B" neurosecretory fibers (Zambrano, 1972). These fibers originate in the nucleus lateralis tuberis (NLT) region of the hypothalamus (Peter, 1970a). Evidence for a hypothalamohypophyseal portal system has been obtained in *Salvelinus fontinalis* (Henderson, 1969), *Heteropneustes fossilis* (Sathyanesan, 1971) and *Carassius auratus* (Peter, 1973).

In teleosts, hypothalamic control of TSH cells is probably inhibitory. Early studies on *Poecilia (Mollienesia) latipinna* surprisingly showed that when a pituitary from one fish was transplanted into the caudal musculature of a recipient fish from the same clone, the TSH cells in the transplant became hyperactive. In addition, thyroidal activity within the recipient was increased (Ball *et al.*, 1963, 1965; Olivereau and Ball, 1966). Subsequent studies in *Carassius auratus* (Johansen, 1967; Peter, 1972) and *Gasterosteus aculeatus* (Leatherland,

1970) demonstrated that ectopic transplantation of the pituitary resulted in increased thyroidal activity. Evidence of hyperactivity was also obtained from histological and radiochemical examination of pituitaries of *Anguilla anguilla* and *Salmo gairdneri* when these were removed from the fish and placed in tissue culture (Baker, 1965, 1969a, 1969b). In *Carassius auratus*, stereotaxic lesioning of the NLT and pituitary stalk increased thyroid activity (Peter, 1970a). Peter (1973) also observed decreased thyroid radioiodide uptake in goldfish injected with fish hypothalamic extracts. Mammalian thyrotropic releasing hormone (TRH) seems to inhibit the activity of the pituitary (Bromage, 1975), but it may be exerting non-specific control over the thyroid axis (Peter and McKoewn, 1975). Thus, one cannot infer that the presumed fish thyrotropin-releasing inhibitory factor (TIF) is structurally similar to TRH. Furthermore, while most evidence favors the presence of a hypothalamic TIF in teleosts, it has been obtained in an indirect manner. Absolute certainty will only come with the purification and elucidation of the structures of both fish TSH and TIF.

As in higher vertebrates, thyroxine exerts a negative feedback at the level of the pituitary. Baker (1965) observed a decrease in the extent of degranulation in thyrotropes of pituitary cultures following the addition to the medium of T_4. Later studies (Baker, 1969a,b) showed that T_4 decreased radiouridine uptake and degranulation in TSH cells *in vitro*. These data suggest T_4 inhibition of both synthesis and release of TSH, although inhibition of one may be affecting the other. Feedback control of T_4 on the pituitary also was demonstrated in the goldfish (Peter, 1971, 1972). The pituitary's responsiveness to this feedback appears to be set by the hypothalamus. This is shown by the transplant studies of Ball *et al.* (1963; see above) in which thyrotropes of

ectopically transplanted pituitaries remained hyperactive after a hyperthyroid state developed. In fish, T_4 seems to be involved in a positive feedback at the hypothalamus which, by augmenting TIF release, further inhibits thyrotropic activity (Peter, 1971).

The Actions of Thyroid Hormones in Teleosts

The existence of a complete thyroid axis responsive to circulating thyroid hormones and subject to neural control probably linked to external environmental receptors suggests considerable importance of thyroid hormones in teleosts. However, uncertainties concerning the physiological importance of thyroid hormones in some teleosts have been expressed (Gorbman, 1969; Etkin and Gona, 1974). It is difficult to discern a discrete role of the thyroid from a review of the literature. Nevertheless, there exists a large body of information for the thyroid's multifarious complicity in teleost physiology (see reviews by Pickford, 1957; Gorbman, 1969).

Much of the confusion concerning the role of the thyroid has arisen from contradictory evidence of the ability of T_4 to stimulate respiration. In higher vertebrates, administration of T_4 elicits a thermogenic response. Recent studies indicate that T_3 increases the activity of Na^+-K^+-ATPase in rats (Edelman and Ismail-Beigi, 1974). This in turn, decreases the ADP/ATP ratio which then stimulates the mitochondrial electron transport system. The high-energy phosphate nucleotide is subsequently replenished at the expense of oxygen.

The role of thyroid hormones in teleost respiration remains an enigma. Administration of T_4, antithyroid drugs, or thyroidectomy usually are not accompanied by changes in the rate of oxygen consumption (Pickford, 1957; Gorbman, 1969; Etkin and Gona, 1974). Yet the

thyroid is known to be involved in events which require energy and increased metabolism. T_4 administration increases swimming and fluttering activity in fish (Hoar *et al.*, 1955; Baggerman, 1962; Sage, 1968; Woodhead, 1970). More significantly, thyroidal activity seems to be increased during exercise (Higgs and Eales, 1971). Radiothyroidectomy of *Aequipes latifrons* resulted in a permanent decrease in oxygen consumption (Ruhland, 1971).

Thyroid hormones have a wide variety of actions controlling daily physiological events (see reviews by Pickford, 1957; Gorbman, 1969). A detailed discussion of these is not warranted here However, it might be worth emphasizing that the net effects of thyroid hormones are extremely dependent on the metabolic state of the animal (see Ingbar and Woeber, 1974). For example, thyroxine given to an immature fish undergoing rapid growth may have a different effect on protein metabolism than T_4 administered to an adult one month before spawning. Similarly, the effects of thyroid hormones on lipid metabolism may differ between a starved and a well-fed fish. As the metabolic state modifies and is modified by the total activity of the endocrine system, the actions of thyroid hormones are thus dependent on changing levels of other hormones (Ingbar and Woeber, 1974 ; Bray and Jacobs, 1974).

Appreciation of the state of the animal in studying T_4 and T_3 effects in fish has been emphasized (Barrington *et al.*, 1961; Narayansingh and Eales, 1975b). Contradictory conclusions on the actions of thyroid hormones exist. For example, the thyroid has been implicated in increasing growth (La Roche *et al.*, 1966), yet thyroid hormone administration has been shown to decrease growth (La Roche and Leblond, 1952). Although T_3 and T_4 seem to enhance oxidative decarboxylation of

radiogluconate (Hochachka, 1962), T_4 reduces the activity of glucose-6-phosphate dehydrogenase (Leray *et al.*, 1970). Additionally, T_4 and T_3 have been implicated in protein synthesis (Narayansingh and Eales, 1975a) or net positive N balance and increased ammonia excretion (Hoar, 1958) which indicates net negative N balance. These apparently conflicting data may be reconciled by closer examination of both the physiological state and the environmental history of the fish.

The Role of Thyroid Hormones in Seasonally Related Events

Seasonal changes in the storage or utilization of energy substrates have important consequences to the physiology of teleosts. Such alterations may permit the fish to adjust to, or take advantage of, a continually changing environment. It is not surprising therefore, to find the thyroid involved in the orchestration of seasonally-occurring events.

The thyroid has been implicated in seasonal adjustments to thermal change (Hoar, 1959) although its activity shows variable response to applied temperature changes (Gorbman, 1969). T_4 administration increases swimming activity (Hoar *et al.*, 1955; Baggerman, 1962; Sage, 1968; Woodhead, 1970), enhances sensitivity to electrical stimulation (Hoar *et al.*, 1955), and diminishes the threshold of sensory-evoked potentials in the brain of teleosts (Gorbman *et al.*, 1964; Hara *et al.*, 1965). Thus, seasonal variation in thyroidal activity may "sensitize" or "prepare" other systems to environmental cues which then predispose the animal to a subsequent seasonal event (see Hoar *et al.*, 1955; Hoar, 1959, 1973).

The thyroid may be involved directly in controlling seasonal events. Using TSH, T_4 and antithyroid drugs, Baggerman (1962) demonstrated

thyroid-dependent seasonal changes of salinity preference in *Gasterosteus aculeatus*. These changes, combined with increased swimming activity (Baggerman, 1962), may then induce migratory behavior in sticklebacks. Using histological and radiochemical indices, Eales (1963) reported seasonal changes in thyroid activity occurring at the time of migration in several species of juvenile Pacific salmon.

Eales (1965) found an increase in thyroid activity at the time of the seasonal parr-smolt transformation in *Salmo gairdneri*. Smoltification, or "silvering", is due to an increased deposition of guanine in the skin. Eales' (1965) observations are supported by the demonstration that T_4 increases the incorporation of radioglycine into epidermal guanine in *Salmo irideus* (Matty and Sheltawy, 1967).

Employing artificial and natural regimes of temperature and photoperiod, Eales (1965) attempted to discern the predominant environmental cue which triggers smoltification. In two-year-old potential smolts, epithelial cell height was found to be responsive only to lengthening photoperiod. Contrary to this, radioiodide metabolism was effected by both temperature and photoperiod.

Swift (1959) observed an annual cycle of thyroidal activity in *Salmo trutta*. Histological indices suggested that thyroid hormone production was inversely correlated with water temperature. Yet the rate of radioiodide clearance from the thyroid reached a peak level in the summer at the time of maximal water temperature. As in Eales' study (1965), Swift's conclusions were hampered by anomalous discrepancies between histological and radiochemical data. It has been noted that histological and physiological criteria frequently provide conflicting assessments of the thyroid's activity (Drury and Eales, 1968).

Thyroid-reproduction Association

Annual cycles of thyroidal activity are correlated with reproductive cycles (see reviews by Matty, 1960; Swift, 1960). Swift (1955, 1959) reported annual cycles of thyroidal activity in both immature and mature *Salmo trutta*. The thyroid in three-year-old brown trout was more active at the time of spawning than in yearlings. Thus, reproductive activity may impose changes on cycles which, in the immature animals, are primarily dependent on external environmental cues. Similarly, Yaron (1969) described histological changes in the thyroid of *Acanthobrama terrae-sanctae* which were correlated with water temperature except at the time of spawning.

Histological studies of the thyroid follicles in *Phoxinus phoxinus* suggested increased thyroidal activity in the months immediately prior to spawning (Barrington and Matty, 1954). This led the authors to speculate that thyroid hormones may stimulate the growth of ova and final maturation of germ cells which begin before high levels of gonadal steroids appear. Thyroidal activity diminishes during the spawning period, presumably when high concentrations of steroids become available. Earlier, Barrington and Matty (1952) found gonadal maturation to be inhibited in fish treated with thiourea. However, thiourea may be exerting this effect through general depression of metabolism (Matty, 1960). Berg *et al.*, (1959) demonstrated a relation between radiothyroxine production and gonadal recrudescence in *Fundulus heteroclitus*.

Thyroid-gonadal relations were studied during the 24-day gestation period of the guppy, *Poecilia reticulata* (Bromage and Sage, 1968; Sage and Bromage, 1970a,b). Thyrotropic cells became active in the first phase of the gestational cycle (Sage and Bromage, 1970a). This

activity, determined by histological criteria, supported previous studies of thyroid secretion in *Poecilia* as measured by colloid interferometry (Bromage and Sage, 1968). Furthermore, females whose ova were not fertilized displayed no cyclic changes of follicular colloid density (Bromage and Sage, 1968).

In intact *Poecilia*, thiourea stimulated activity in the gonadotropes whereas thyroxine inhibited them. In males, estrogen stimulated the thyrotropes *in vivo* and methyl testosterone inhibited them. Reciprocal feedbacks were also observed *in vitro*, although, unlike the *in vivo* study, both steroids inhibited thyrotropes from males and females (Sage and Bromage, 1970b). The opposing effects of the gonadal steroids in males *in vivo* is not understood. Nevertheless, these studies imply the intriguing possibility that feedback systems and thus, endocrine axes, are not entirely discrete in teleosts.

The control of the thyroid is related to gonadal control in both higher vertebrates (Bray and Jacobs, 1974) and in teleost fish (Sage, 1973). The studies in *Poecilia* cited above (Bromage and Sage, 1968; Sage and Bromage 1970a,b) provide not only strong evidence for a thyroid-gonadal association, but a causal link via reciprocal feedback controls at the hypophyseal level. The anatomical proximity of the pituitary thyrotropes and gonadotropes (Sage and Bern, 1971) and the striking primary structural similarity among mammalian pituitary glycoproteins, TSH, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) (Pierce *et al.*, 1971; Papkoff, 1972), lend credence to the postulate of a phylogenetically-old relation between thyroidal and gonadal control systems (Sage, 1973; Hoar, 1973).

The Pineal and Sensitivity of Vertebrate Endocrine Systems to Photoperiod

The reproductive cycles of many temperate zone vertebrates are regulated by seasonal changes in daylength. In considering the mechanisms by which light may influence reproduction and possibly, thyroidal activity, the pineal organ immediately becomes suspect. The pineal is photoreceptive in all vertebrates (Ralph, 1975). In mammals, much evidence exists for an endocrine role of the pineal which is controlled by noradrenergic, cyclic nucleotide-mediated, innervation originating in the optic nerve (Wurtman and Cardinali, 1974). Hence, the term "neuroendocrine transducer" has been applied to describe the pinealocyte of higher vertebrates (Wurtman and Axelrod, 1965).

One form of endocrine output of the pineal in higher vertebrates is probably the indole derivative, 5-methoxy-N-acetyltryptamine or melatonin (5-MAT). The pineal is one of the few organs (other than the retina and the Harderian gland) which possesses the enzymes required for the biosynthesis of 5-MAT from tryptophan (Cardinali, 1974). Activities of the enzymes responsible for the last two steps in the synthetic pathway, serotonin-N-acetyl transferase and hydroxyindole-O-methyl transferase (HIOMT), are augmented by darkness-induced neuronal input and inhibited by feedback from gonadal steroids (see review, Cardinali, 1974). 5-MAT is best known for its antigonadotropic effect (Reiter, 1973) although it has been shown to affect other endocrine axes (see Cardinali, 1974) including the thyroid (Baschieri *et al*, 1963; Panda and Turner, 1968). Melatonin administration produces opposite effects to those of pinealectomy (see Reiter, 1973; Cardinali, 1974).

Evidence for an endocrine role of the pineal in teleosts has recently been obtained (Fenwick, 1970a,b; Urasaki, 1973; de Vlamming, 1975). HIOMT activity was reported in *Salmo gairdneri* (Quay, 1965) and melatonin itself was isolated and measured spectrophotofluorometrically in Pacific salmon, *Oncorhynchus tshawytscha* (Fenwick, 1970a). In support of an antigonadotropic function, levels of pineal melatonin were found to be higher in immature salmon (1200 ng/pineal) than in mature salmon (180 ng/pineal) (Fenwick, 1970a). Thus, in response to a changing photoperiod, the pineal may influence seasonal endocrine cycles in teleosts, possibly via the hormone, 5-MAT.

Direction of Thesis Research

In summary, control of the thyroid gland in teleost fish seems to be as sophisticated as that of higher vertebrates. Furthermore, thyroid hormones regulate, in part, many aspects of day-to-day physiological events. Changing levels of thyroid hormone production, in conjunction with possible alterations in sensitivity of cells to thyroid hormones, appear to be involved in long-term control of seasonally-related physiological functions. In nonmigratory, temperate zone, adult teleosts, the reproductive cycle, climaxing in the spawning period, is the predominant annual event.

The purpose of the present study was to examine the possibility of annual changes in the circulating levels of the thyroid hormones, T_3 and T_4, in the brook trout, *Salvelinus fontinalis* (Mitchill). In the light of postulated thyroid-gonadal associations, attention was directed to the plasma concentrations of thyroid hormones in relation to the annual reproductive cycle. Also, an attempt was made to examine

the concentration of pineal melatonin at various times of the year.

The index of thyroidal activity employed was the measurement by radioimmunoassay (RIA) of the concentrations of circulating thyroid hormones. RIA has the advantages of extremely high sensitivity, high specificity and high reproducibility. Unlike thyroid follicle histology, colloid interferometry, radioiodide uptake or clearance by the thyroid, and protein-bound or butanol-extractable iodide, RIA enables one to examine changes in the circulating levels of T_3 and T_4 separately. Also, as plasma hormone concentrations result from the net synthesis of all contributing follicles, they are not affected by variations in response and activity of anatomically separate follicles (Chavin, 1956; Chavin and Bouwman, 1965; Peter 1970b).

To date, few analyses have been made of the circulating levels of thyroid hormones in teleosts employing saturation binding techniques. The data are limited to observation of single or a few samples from 12 species (Refetoff *et al.*, 1970; Higgs and Eales, 1973; Brown and Eales, 1976).

The brook trout was chosen for the study because its physiology is well-known. The reproductive cycle has been described and there is evidence to indicate that it is normally regulated by seasonal changes in daylength (Henderson, 1962, 1963). The time of spawning can be advanced or retarded by several months using artificial photoperiods. This enables one to examine thyroidal activity during a photoaccelerated reproductive cycle which could provide further evidence for a thyroid-gonadal association.

Materials and Methods

Annual Cycle Study

Brook trout used in the annual cycle study were obtained from and held at the Raven Rearing Station, Caroline, Alberta (52n, 114 5w). They were confined in a natural, spring-fed pond by crosscurrently-placed, underwater fencing. The pen measured approximately 10x8x1.5 m. In addition to brook trout, the pen held adult rainbow trout (*Salmo gairdneri*) together with a few brown trout (*Salmo trutta*) and Rocky Mountain whitefish (*Prosopium williamsoni*).

The fish received a prepared food twice daily at 0900 and 1600 hr (Silver Cup #4, Murray Elevators, Murray, Utah). However, feeding could not be controlled entirely. Evidence of cannibalism was found occasionally and it is reasonable to assume that the fish derived some nutrition from naturally available food sources.

At approximately monthly intervals, a sample of six adult brook trout was taken from the pond between July, 1975 and June, 1976. Sampling was always performed between 1030 and 1200 hr to minimize variation that might be introduced from possible diurnal fluctuations in hormone levels. The fish were collected by seining the pond. This was done with care so as not to excite the fish. Trout were removed from the seine purse individually and immediately transferred to a bucket containing 0.005% tricaine methane sulfonate (MS222 - Sandoz Pharmaceuticals, Dorval, Quebec) in pond water. Once the fish was lightly anesthetized (2-3 min), blood was collected using a sterile, 5 ml, heparinized syringe fitted with a 22ga, 3.8 cm needle. Blood was taken from the vessels in the hemal arch in the area of the caudal peduncle. The blood sample was kept on ice in a 15 ml centrifuge tube.

The fish was weighed and measured for standard length. The gonads were dissected, weighed and placed in Davidson's fixative (Henderson, 1963). The fish was decapitated and the head placed over solid CO_2 for later dissection of the pineal.

As soon as six fish had been processed in this manner (1.5 hr), the blood samples were centrifuged at 2000 x G for 15 min. Usually, 1 to 2 ml plasma was obtained from each fish. From each blood sample, 2 aliquots of plasma were collected and stored in polypropylene, snap-cap tubes. The plasma samples were immediately frozen over solid CO_2 for the return drive to Calgary. At Calgary, they were stored at -80^0 for periods of a week to three months. One aliquot was used for T_4 measurements and the second for T_3 to avoid repeated thawing and re-freezing during the assays.

On June 29, 1976, two additional samples (n=4 in each sample) were taken at 0530 and 2030 hr. The three samples collected on this date were used to investigate the possibility of diurnal changes in circulating levels of T_3 and T_4.

Accelerated Photoperiod Group

An experiment was carried out in which fish were exposed to an accelerated light regime that is known to advance the time of functional maturity by three to four months in adult brook trout (Henderson, 1963). A group of 24 brook trout was obtained for us by the Regional Biologist at Red Deer, Alberta in mid-February, 1976. These were captured by electrofishing from Stauffer Creek, approximately 5 km northwest of the Raven Station. The trout were transferred to a tank at the Raven Station within one hour of capture. The fish were

held in a circular, center draining, fiberglass tank 1.8 m in diameter and 0.9 m deep. The tank was supplied with water by tapping the run off of springs which fed the pond. Polyvinyl chloride pipes and fixtures were used for all plumbing.

The tank was equipped with a lightproof, plywood cover containing a central panel of lucite measuring 0.6 by 0.7 m. A box housing four fluorescent lights was fitted over the lucite panel. This was protected by an "A frame" plywood cover. The length of the daily light period was controlled by a time clock. Intensity of illumination at the water surface was measured photometrically (Model J16 Digital Photometer, Textronix Inc., Oregon) and varied from 5000 lx at the center to 30 lx at the periphery.

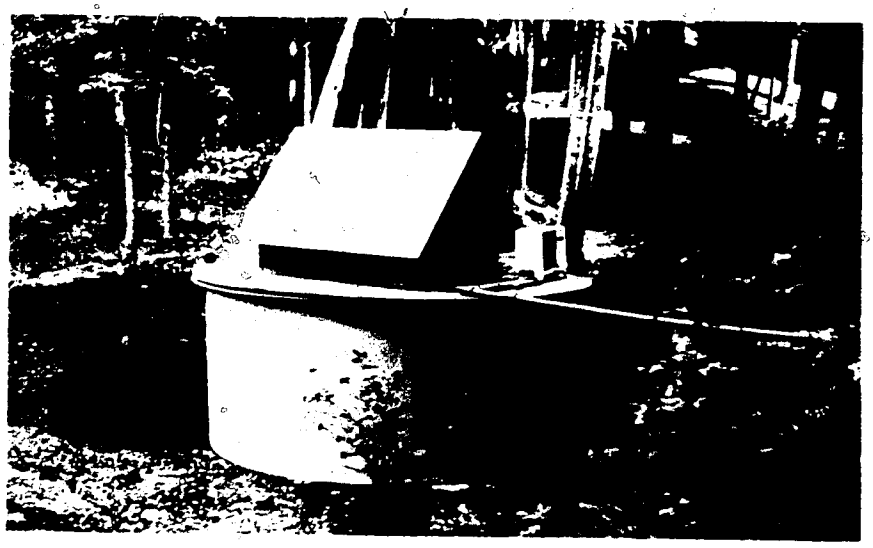
The brook trout were placed in the tank on Feb. 25, 1976 and exposed to natural daylengths for one week by removing the light housing and cover. On March 6, the artificial lights were replaced on the cover and the fish exposed to a 7L:17D regime. The light phase was increased by one hour each week until 21L:3D was reached. This regime was maintained for four weeks, after which the photoperiod was decreased by one hour per week to 7L:17D. Increase or decrease of the photoperiod was done alternately in the morning one week and in the evening the following week.

Sampling of this group began April 29, 1976. Four fish were taken at monthly intervals and treated in a manner identical to that used in the annual cycle study. The pond and experimental tank are illustrated in Figure 1.

Reproductive Cycles

Reproductive activity of the fish was monitored by measuring the

A



B

Figure 1. Holding facilities at the Raven Rearing Station, Caroline, Alberta. Photograph A shows the pens constructed in a natural pond. The pen in the foreground was used for the annual cycle study. Photograph B shows the tank used for the acceleration photoperiod study. Both the tank and pond were fed by springs located in the bank seen in the background of photograph A.

average diameter of ova (procedure of Henderson, 1963) and the gonosomatic index (GSI). This index is calculated by the following equation:

$$GSI = \frac{\text{weight of gonads}}{\text{weight of fish}} \times 100$$

Water Temperature and Iodide Content

Water temperatures in the pond and tank were monitored continuously by a temperature recorder (Rigosha, Tokyo). The monthly minimum and maximum temperatures were noted from the record and logged.

Samples of pond water were taken on each sampling day from January to June in 1976. The water was stored in dark, glass-stopped bottles at 4° until assayed. Total iodine concentration was measured spectrophotometrically using the Hycell cuvette PBI procedure (Hycell Inc., Houston, Texas). In all samples, the iodine concentration was found to be less than 0.5 µg/l.

Radioimmunoassays for T_3 and T_4

Initially, the Mallinckrodt RIA-MAT kit RIA for T_3 (Mallinckrodt Chemical Works, Pt. Claire, Quebec) was tested for its ability to measure T_3 in the plasma of rainbow trout, *Salmo gairdneri*. The procedure recommended by Mallinckrodt was followed. Difficulties with this assay arose from the finding that up to 40% of radiolabelled T_3 ($*T_3$) existed in the presumed "bound" fraction in samples which were devoid of antibody and supposedly had the "free" $*T_3$ removed. Several modifications were made in the Mallinckrodt protocol in an attempt to reduce this non-specific or false "binding" value. These are described in Appendix I. Briefly, the various revisions which were employed did not

significantly reduce the "binding" or they did not give consistent results. Consequently, the Mallinckrodt assay was abandoned.

In the fall of 1975, Dr. J. G. Eales of the University of Manitoba made his procedure for measuring both T_3 and T_4 on Sephadex gel columns available to us. Brown and Eales (1976) made a detailed assessment of the quality of their assay. They found "within" and "between" assay reproducibilities to be satisfactory and they reported mean detection limits of 12.5 ng% and 9.5 ng% for the T_4 and T_3 assays respectively. The procedures of Brown and Eales (1976), with minor modifications, were utilized throughout the study and are summarized in Appendix II.

Standard solutions were made using T_3-salt and T_4 (Sigma Chemical Co., St. Louis, Mo.). 100 µg T_3 or T_4 was weighed to the nearest microgram (M5 Microgramatic, E. Mettler, Zurich). Each was added to 0.1 N NaOH in a 1 l volumetric flask (all glassware was siliconized). The stock solution (100 ng/ml) was mixed for at least 30 minutes before preparing working standards. The stock solution was stored at 4^0 for periods up to one month. Working standards were prepared prior to assay in 100 ml volumetrics at concentrations of 0, 1, 2, 3 and 4 ng/ml. These were stored at 4^0 for up to one week.

Radioactive-labelled hormone ($[^{125}I]T_3$ and $[^{125}I]T_4$) was obtained in 100 µCi lots (K and T Biological Services, Edmonton, Alberta). The specific activity of $*T_3$ was approximately 550 mCi/mg and of $*T_4$ approximately 700 mCi/mg. Five µl aliquots of the labelled hormone were pipetted into 2.5 ml 0.1 N NaOH in 3 ml, screw-cap vials. These were kept in lead containers at -20^0 for up to two weeks. Each was thawed and diluted ten fold immediately prior to assay (final activity was approximately 3000 dpm/100 µl).

Antibody to T_3 (anti-T_3) and T_4 (anti-T_4) was obtained from K and T Biological Services in lyophilized form. These had ten times the titre normally supplied by K and T for clinical assays. As described by Brown and Eales (1976), the anti-T_3 and anti-T_4 were reconstituted in cold buffer (22.5 ml for anti-T_3 and 30 ml for anti-T_4). Antibody dilution was further adjusted at the time of assay to give a sensitive standard curve with 40-60% binding of $*T_3$ or $*T_4$ at 0 ng/ml standard. The final dilution was usually 1:450 (antibody:buffer) for anti-T_3 and 1:600 for anti-T_4.

The columns used were 5 ml syringe barrels packed with Sephadex G-25 fine (*Sephadex* is a trademark of Pharmacia Fine Chemicals, Dorval, Quebec). Total gel volume was approximately 1.5 ml. A rack was made to accomodate sixty columns.

Samples were counted for 10 min each on an automatic gamma counter (Nuclear Chicago) equipped with a 3-inch crystal. The counter had an efficiency of 83%.

The assay procedure followed is that of Brown and Eales (1976) with the following exceptions:

(1) Brown and Eales (1976) used 0.075 M barbital buffer, pH 8.6 in both the T_3 and T_4 RIAs. We found, as has Dr. Baynton of the Foothills Hospital, Calgary, that the anti-T_3 binding is partially inhibited by barbital. Therefore, Dr. Baynton's procedure (pers. comm.) employing 0.1 M sodium phosphate buffer, pH 7.4 was used in the T_3 RIA.

(2) After preliminary testing of several hundred samples, it was found that percent recovery of either T_3 or T_4 after extraction was consistently ninety-eight to one hundred percent. Thereafter, the initial rinse was discarded with an appreciable saving of counting time.

Plasma samples (25-200 µl) from each fish were assayed in triplicate. The first three standards (0,1 and 2 ng/ml) were assayed in quadruplicate, as they covered the range of hormone concentrations for most samples. The other standards (3 and 4 ng/ml) were done in triplicate. Some plasma samples were measured in two separate assays on consecutive days. Crossreactivity was evaluated by testing the ability of 3 ng T_4 to displace $*T_3$ from anti-T_3 and the ability of 10 ng T_3 to displace $*T_4$ from anti-T_4.

Thyroid Hormone-Protein Binding

After finding lower levels of T_4 than those of T_3 in the first samples assayed (see Results), it was decided to examine qualitatively the relative binding of T_3 and T_4 to TBP in plasma samples of brook trout. This was done by allowing trace amounts of $*T_3$ and $*T_4$ to equilibrate with plasma proteins. The bound fraction was separated from the free by column chromatography on Sephadex *G*-25 gel. This method can discriminate, to a certain extent, between weak association with and strong binding to plasma proteins. This is due to the fact that the ether linkages in the low water regain gels (up to *G*-50) weakly "bind" phenolic derivatives (e.g., T_3 and T_4) (Determann and Walter, 1968).

The specific procedure was as follows:

(1) 100 µl of eight plasma samples of brook trout from the annual cycle study was pipetted into 10x75 mm test tubes (*plasma* tubes).

(2) 100 µl of $*T_3$ or $*T_4$ (approximately 10,000 dpm) prepared in 0.1 M sodium phosphate buffer, pH 7.4 was added to each of the *plasma* tubes.

(3) 100 µl of the same preparation of $*T_3$ or $*T_4$ was pipetted

into four tubes of *total counts*. Also, 100 µl of the same was added to two tubes with 100 µl buffer (*iodide* tubes).

(4) After mixing gently, the *plasma* and *iodide* tubes were incubated for 4 hr at 4^0.

(5) 100 µl from each tube was pipetted onto the columns used in the RIA (see above) previously washed with 0.1 M sodium phosphate buffer, pH 7.4. The sample was run into the column ahead of 2 ml buffer.

(6) The eluant was collected, mixed, and counted for 10 min.

(7) The percentage of bound radiolabelled hormone was calculated by the following equation:

$$\%B = \frac{(Ep - Ei)\ 200}{TC - Ei}$$

where Ep is the radioactivity (DPM) in the eluant from *plasma* tubes, Ei is the average radioactivity (DPM) in the eluant from the two *iodide* tubes, and TC is the average DPM in the four *total count* tubes.

In order to validate the procedure and have a reference point for comparing the teleost data, T_3-TBP and T_4-TBP binding was tested in plasma samples from two mammalian species. The procedure above was used except for an incubation for 2 hr at 20^0.

Spectrophotofluorometric Assay for Melatonin

All glassware for 5-MAT extraction was washed with concentrated nitric acid and rinsed ten times with triple-distilled water. Melatonin standard (Mann Assayed Quality, Schwarz/Mann, Orangeburg, New York) was prepared by weighing 1 mg (to the nearest µg) and mixing it in 1000 ml 0.1 N HCl, 0.5% ascorbic acid (ascorbic acid obtained from Nutritional Biochemical Corp., Cleveland, Ohio). Serial dilutions of the stock

standard were made immediately prior to assay. P-cymene (J.T. Baker Chemical Co., Hayward, Calif.) was purified according to the distillation method of Quay and Baker (1965).

Melatonin was extracted in alkaline conditions with p-cymene according to the procedure of Quay (1963). Extracted 5-MAT was measured on an Aminco-Bowman spectrophotofluorometer equipped with an X-Y recorder (American Instrument Co., Silver Spring, Maryland). 100 µl of the final aqueous phase (see Quay, 1963) was transferred to a quartz micro-cuvette (capacity 0.2 ml). 50 µl concentrated HCl was added to ensure fluorescence at 550 nm (Udenfriend *et al.*, 1955). The sample was excited with monochromatic light at a wavelength of 295 nm.

Standard 5-MAT (1.0 µg/ml) was first extracted and its relative intensity compared to unextracted standard in order to determine extraction efficiency. Next, 0.5 ml 5-MAT standard solution (1.0 µg/ml) was added to 0.5 ml homogenate of three pineals from rainbow trout, *Salmo gairdneri*. This was mixed and 100 µl extracted and assayed. Again, the relative intensity was compared to that of unextracted standard.

Pineals were dissected, pooled, and homogenized from immature rainbow trout (n=4), mature rainbow trout (n=3), and monthly samples of brook trout from the annual cycle study (n=6). Heads were allowed to thaw immediately prior to dissection. Pineals were exposed by removing a flap of skin and bone from the top of the head. The pineal body was gently aspirated into a modified pasteur pipette and lifted slightly to expose the stalk. The stalk was severed at its base with forceps and the pineal transferred to a 5 ml, glass tissue homogenizer containing 0.1 ml 3% ascorbic acid in 1% disodium EDTA and 0.4 ml 0.02 N HCL

saturated with KCl (see Fenwick, 1970a). The homogenizer was weighed before and after the addition of the pineals in order to obtain the weight of a pooled sample. After homogenization, 100 µl was removed and extracted for assay.

Results

Typical standard curves for the T_3 and T_4 RIAs are given in Figure 2. The range from 100-200 pg was the most sensitive part of both standard curves. The volume of each plasma sample was adjusted so that the amount of hormone in the sample fell within or close to this range.

Cross reactivity of anti-T_4 with T_3 and of anti-T_3 to T_4 was found to be less than 1%. This agrees with the analysis made by Brown and Eales (1976) and confirms the high specificity of both antibodies reported by the supplier (described in Brown and Eales).

Annual Cycle Study

Total plasma concentrations of the thyroid hormones together with data on the size and sex of fish sampled in the annual cycle study are reported in Table 1.

From Table 1, it is evident that circulating levels of T_4 varied markedly throughout the year. Mean T_4 values displayed a fourfold change over the year, ranging from a minimum of 0.8 ng/ml in November to a maximum of 3.7 ng/ml in April.

The profile of total plasma T_4 concentration from July, 1975 to June, 1976 is shown in Figure 3. During the summer months of 1975, the levels of T_4 remained fairly constant at about 1.9 ng/ml. Subsequently, the levels dropped precipitously to a concentration of 0.8 ng/ml in early November at the time of spawning. Thereafter, the T_4 titres rose gradually during the late autumn and winter months. In early spring, the T_4 levels exhibited an abrupt increase to 3.7 ng/ml. This was followed by a decrease in late spring and a return to the previous

Figure 2. Typical standard curves for T_3 and T_4 RIAs. Percent bound (percent B) of radioactively-labelled hormone ($*T_3$ or $*T_4$) to the corresponding antibody is plotted against increasing amount of unlabelled, standard hormone (T_3 or T_4). Assuming 100% recovery of hormone after protein extraction (see text), the percent B was calculated by the equation:

$$\text{percent B} = \frac{\text{DPM bound}}{\text{DPM total-DPM iodide}} \times 100$$

Hormone concentrations in plasma samples were determined by reference to the line describing the relation between percent B and hormone concentrations of standard solutions.

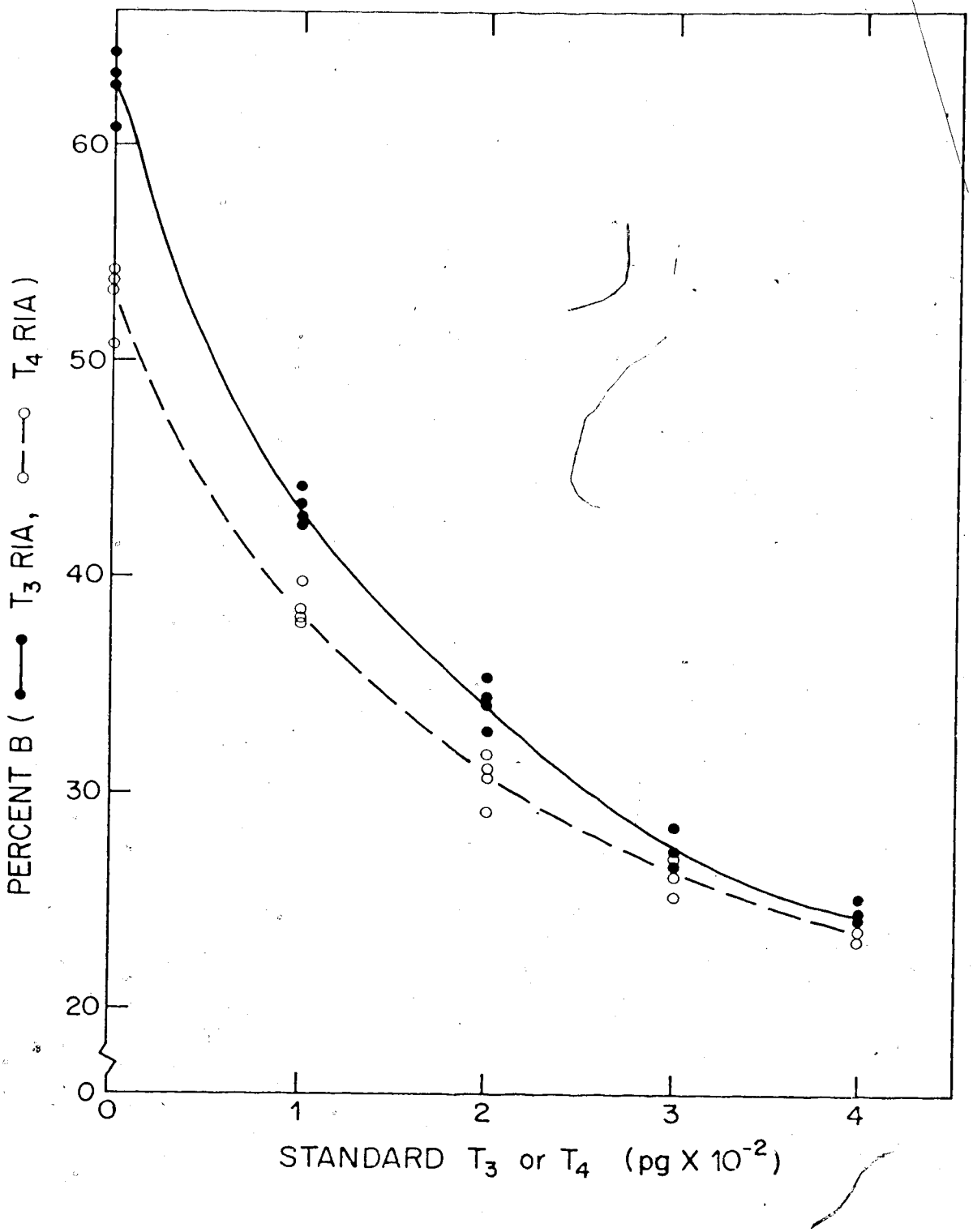

PERCENT B (●—● T_3 RIA, ○—○ T_4 RIA)
60
50
40
30
20
0
0
1
2
3
4
STANDARD T_3 or T_4 (pg X 10^{-2})

TABLE 1. Data from annual cycle study. N=6 for each sampling date. All values given as mean ± 1 SEM. Range is given in brackets.

Sample date[1]	Weight (g)	Standard length[2] (cm)	Sex	T_4 (ng/ml) plasma	T_3 (ng/ml) plasma
Jul 23	251±24 (153-348)	24.6±2.7 (21.5-27.0)	4M, 2F	1.8±0.2 (1.3-2.3)	7.5±1.0 (5.2-9.6)
Aug 21	218±34 (154-372)	23.2±0.5 (22.0-24.9)	4M, 2F	2.1±0.5 (1.6-4.2)	4.9±0.6 (2.6-6.6)
Oct 1	209±45 (90-373)	20.5±4.3 (18.6-29.5)	4M, 2F	1.8±0.1 (1.4-2.5)	3.5±0.8 (1.3-7.0)
Nov 5	150±11 (105-173)	22.1±0.6 (19.5-23.0)	3M, 3F	0.8±0.1 (0.6-1.3)	2.4±0.6 (0.9-4.2)
Dec 18	292±22 (261-401)	23.7±0.7 (22.3-26.9)	3M, 3F	1.2±0.1 (0.7-1.5)	5.6±0.7 (3.0-6.8)
Jan 19	306±11 (260-336)	26.0±1.5 (21.5-30.1)	4M, 2F	1.6±0.1 (1.3-2.0)	6.7±0.8 (4.8-9.7)
Mar 6	162±7.5 (141-185)	23.2±0.4 (22.0-24.6)	3M, 3F	1.7±0.2 (1.3-2.7)	6.2±1.0 (3.2-8.7)
Apr 29	250±57 (149-408)	24.4±1.2 (20.1-29.3)	4M, 2F	3.7±0.3 (2.9-4.7)	8.6±1.0 (5.7-11.5)
May 30	219±18 (177-305)	24.4±0.7 (22.7-27.7)	3M, 3F	3.2±0.1 (2.9-3.5)	6.2±0.1 (5.5-6.5)
Jun 29	199±14 (140-240)	23.9±1.1 (21.9-24.9)	4M 2F	2.1±0.2 (1.6-2.6)	5.5±0.6 (3.4-6.9)

[1]July 23 - December 18 samples refer to 1975. January 19 - June 29 refer to 1976.

[2]Standard length is the distance from the most anterior part of the head to the end of the vertebral column.

summer values of 1.9 ng/ml.

Regression analyses of data from every fish from the annual cycle study and of values within each monthly sample showed no correlation between total T_4 concentration and weight or between T_4 levels and standard length. Additionally, the levels of T_4 were not correlated with sex.

Total plasma T_3 concentrations for each monthly sample in 1975 and 1976 are reported in Table 1 and illustrated in Figure 4. Like T_4 levels, T_3 concentrations varied with time of year, reaching a minimal value in early November and a maximal value in early spring. T_3 levels ranged from 2.4 ng/ml in November to 8.6 ng/ml in April.

Two differences are apparent between the annual pattern of circulating levels of T_3 and T_4. (1) Whereas T_4 levels were more or less constant over the summer and decreased during the month prior to spawning, T_3 levels diminished progressively during the summer and early autumn months to reach their nadir in November. (2) Following spawning, levels of T_3 rose gradually from November to mid-spring in contrast to T_4 levels which exhibited a major increase during early spring.

Regression analyses showed no correlations between T_3 levels and fish size (length or weight). Student's t test showed there was no statistically significant difference between T_3 levels in male and female trout.

Figure 5 presents the iodothyronine concentrations as a ratio of T_3 to T_4. This ratio emphasizes the fact that total plasma T_3 levels in brook trout were consistently 2-6 times greater than those of T_4. In fact, of the 60 animals examined in the annual cycle study, every fish except one had more circulating T_3 than T_4.

Figure 3. Circulating total T_4 concentrations as measured by RIA in *Salvelinus fontinalis* over a twelve month period. Each point represents the mean ± 1 SEM of triplicate analyses of plasma samples from six trout. The vertical bar denotes time of spawning.

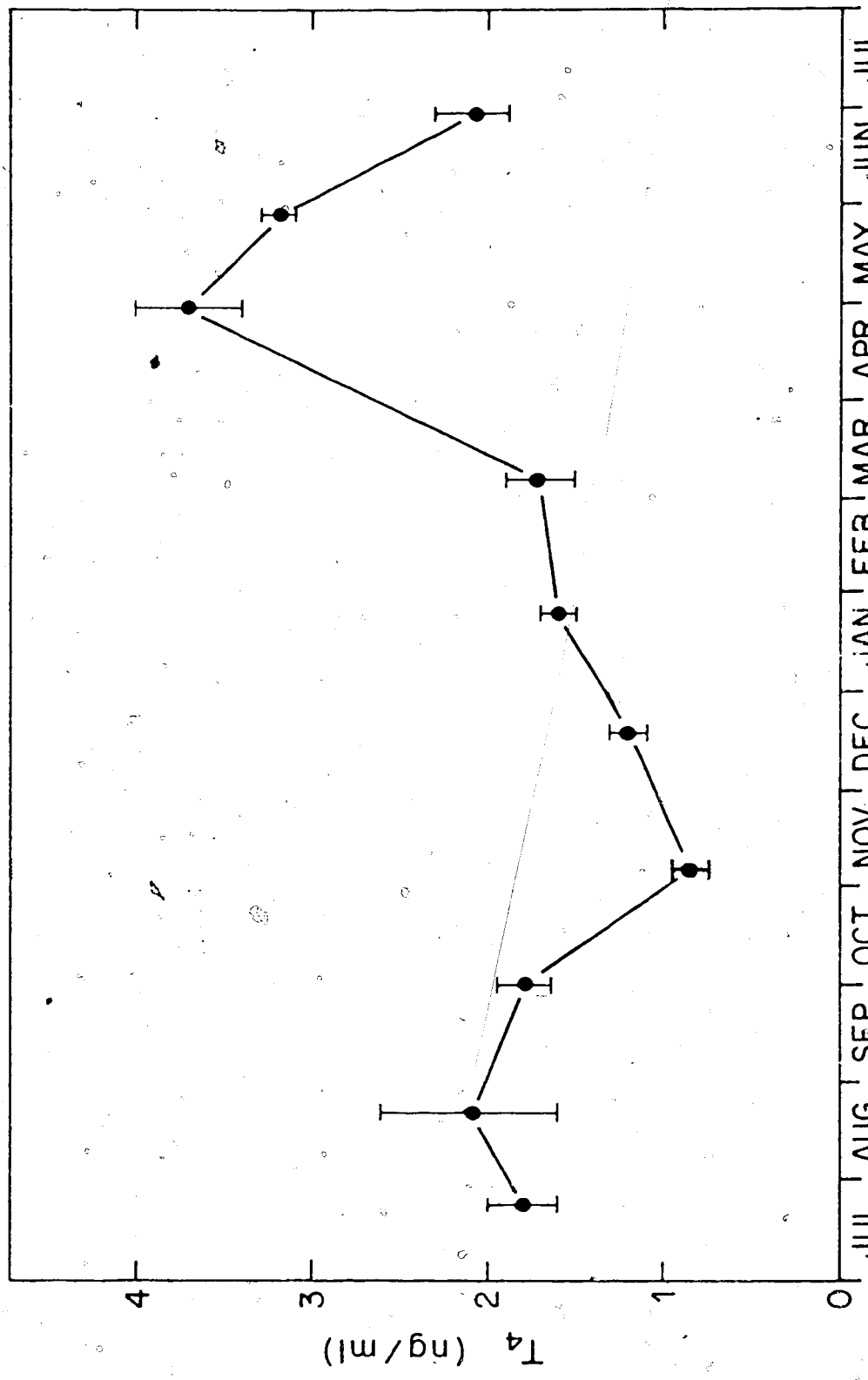

T_4 (ng/ml)
0
1
2
3
4
JUL.
AUG.
SEP.
OCT.
NOV.
DEC.
JAN.
FEB.
MAR.
APR.
MAY
JUN.
JUL.
1975
1976

Figure 4. Circulating total T_3 concentrations as measured by RIA in *Salvelinus fontinalis* over a twelve month period. Each point represents the mean ±1 SEM of triplicate analyses of plasma samples from six trout. The vertical bar denotes time of spawning.

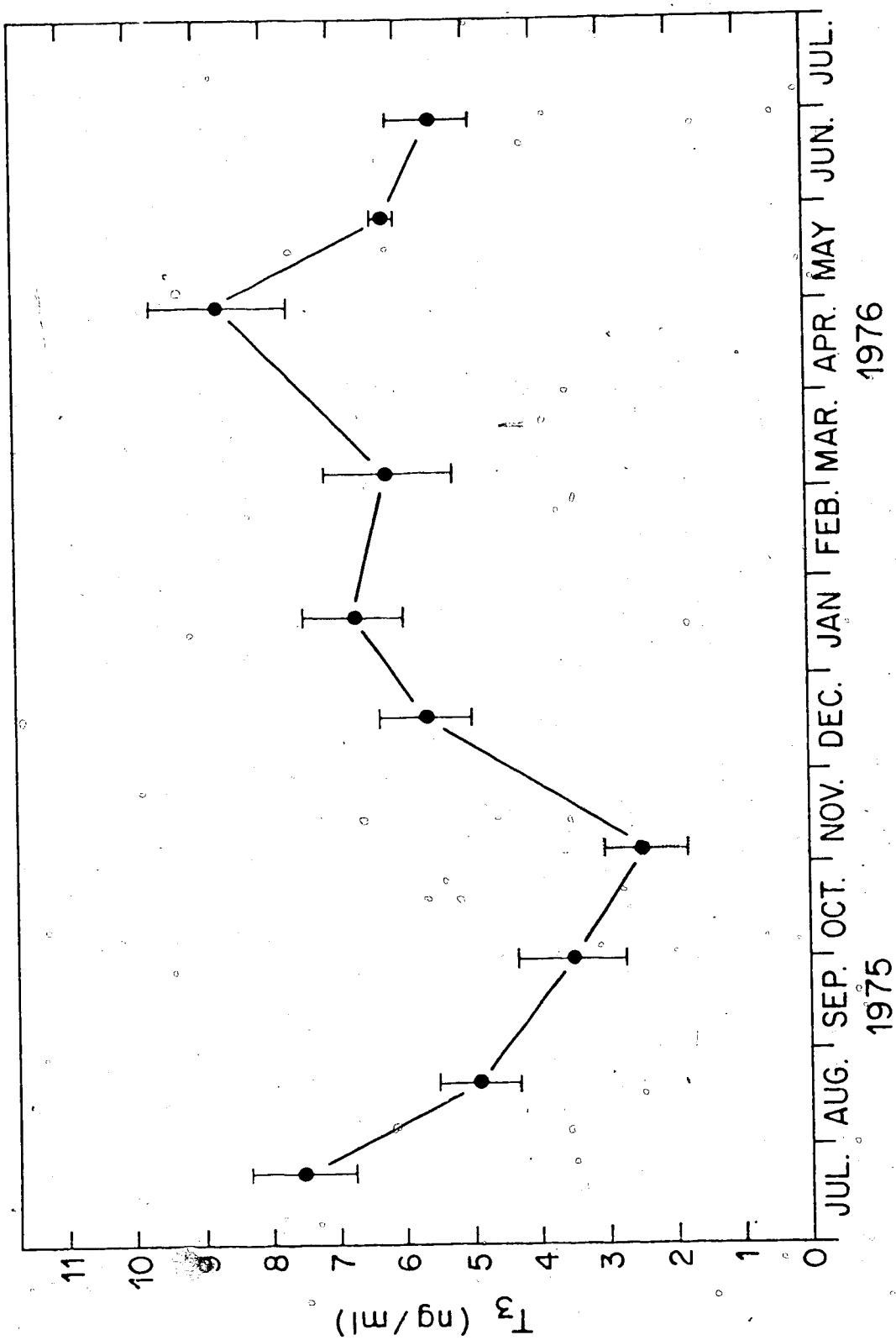
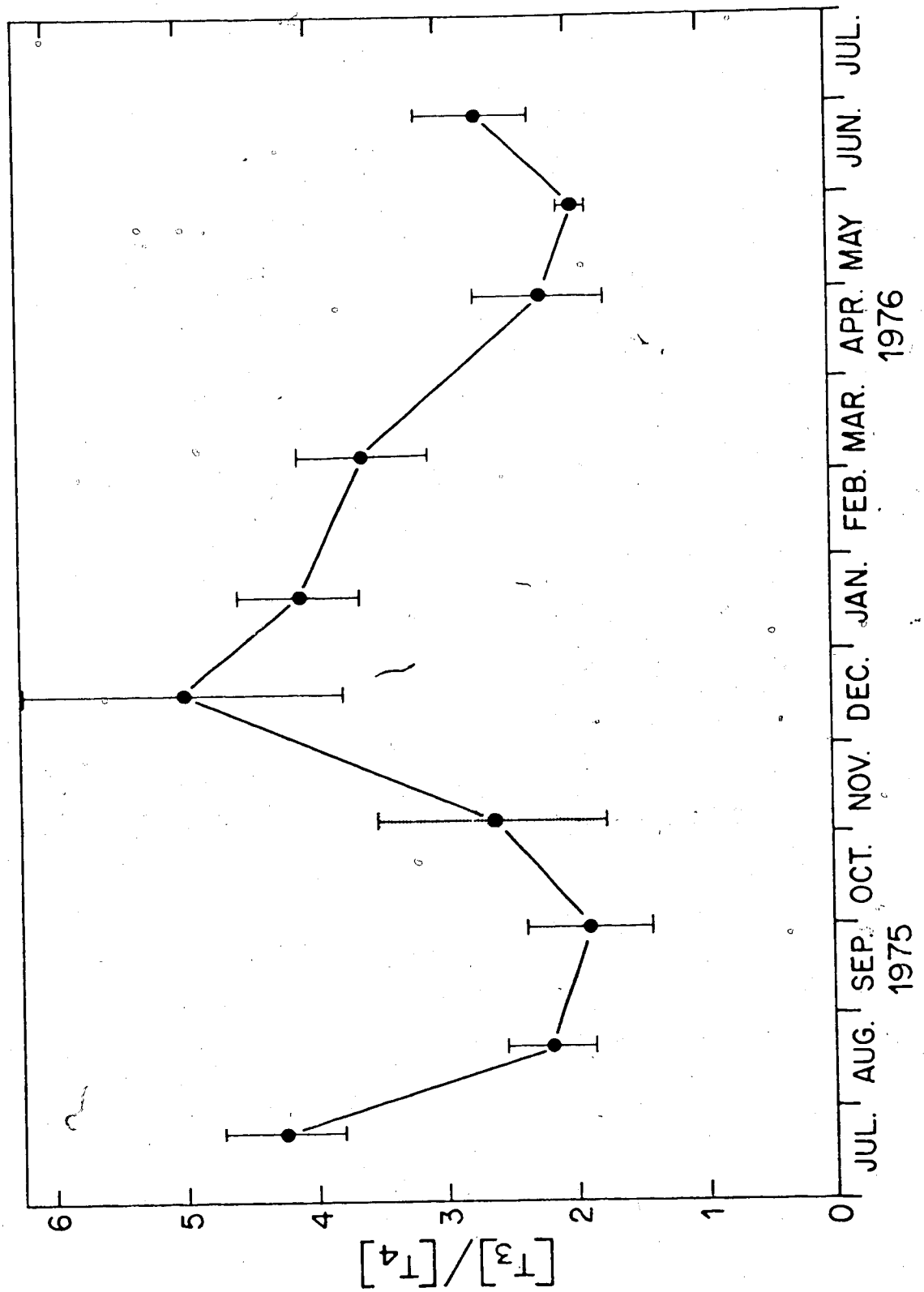

T_3 (ng/ml)
0
1
2
3
4
5
6
7
8
9
10
11
JUL.
AUG.
SEP.
OCT.
NOV.
DEC.
JAN
FEB.
MAR.
APR.
MAY
JUN.
JUL.
1975
1976

Figure 5. Ratio of circulating total T_3 to circulating total T_4 in *Salvelinus fontinalis*. The ratio was calculated for each fish within a monthly sample (n=6). Each point represents the mean ±1 SEM. The vertical bar denotes time of spawning.


[T3]/[T4]
0
1
2
3
4
5
6
JUL.
AUG.
SEP.
OCT.
NOV.
DEC.
JAN.
FEB.
MAR.
APR.
MAY
JUN.
JUL.
1975
1976

The reproductive cycle, as measured by the ova diameter and GSI of males and females, is shown in Figure 6. When the study began in July, 1975, the ova had just entered the secondary growth phase. This is characterized by a rapid increase in ova size, loss of spherical shape of the ova due to crowding, and by the ova becoming increasingly yellow due to the onset of vitellogenesis. Although histological examination of the testes was not done, they were probably in the first stage of rapid spermatogenesis (Henderson, 1962, 1963). When the fish were sampled on November 5, they were functionally mature. The fish were extremely sluggish at this time and eggs and milt were easily expressed. The data from December, 1975 to June, 1976 represent the development of the succeeding year class of gametes that would attain functional maturity in the autumn of 1976.

Daily Variations in Thyroid Hormone Levels

The total plasma T_3 and T_4 levels in fish sampled at different times during the day on June 30, 1976 are presented in Table 2. T_4 titres were low in the 0530 hr sample (0.5±0.1 ng/ml) in comparison to the two other samples taken on the same day. By 1130 hr, T_4 concentrations increased significantly ($p<0.05$) to 2.1±0.2 ng/ml.

Like T_4, T_3 values were lowest in the early morning (4.7±0.4 ng/ml). Unlike T_4, T_3 concentrations rose less dramatically by 1130 hr (5.5±0.6) and apparently continued to increase to reach 6.5±0.7 ng/ml by 2030 hr. Thus, the pattern of daily T_3 fluctuation seems to be a gradual increase from early morning to evening. However, the difference between the 0530 hr sample and 2030 hr sample was not significant ($0.1>p>0.05$).

Figure 6. The reproductive cycle in *Salvelinus fontinalis* as monitored by ova diameter and female and male GSI. The numbers of male and female fish in each sample are given in Table 1. On November 5 (vertical bar), the fish had reached functional maturity. No GSI values are given for the November sample, as spawning had commenced with loss of gametes.

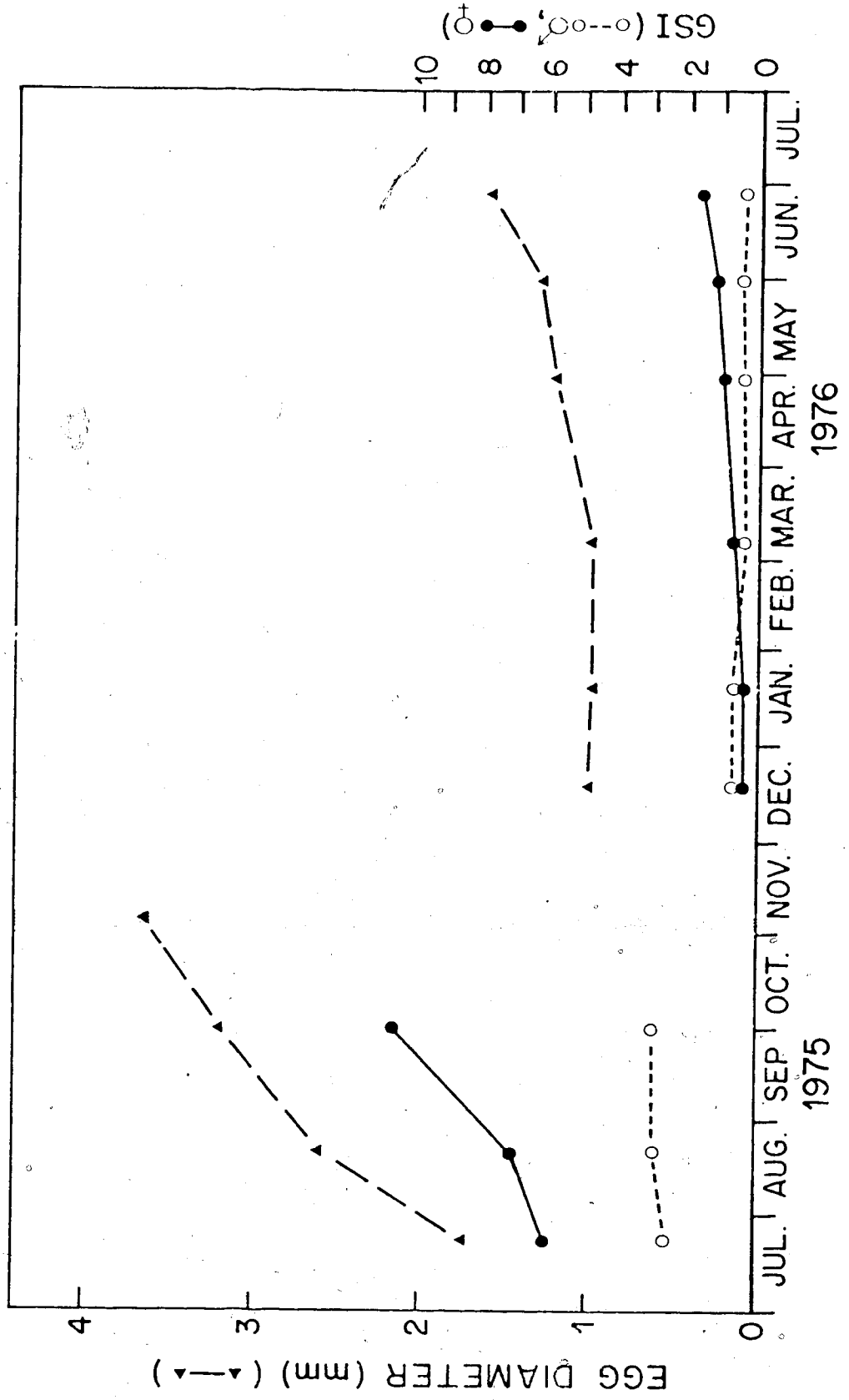

GSI (♀●—●, ♂○--○)
0
2
4
6
8
10
EGG DIAMETER (mm) (▲—▲)
0
1
2
3
4
JUL.
AUG.
SEP
1975
OCT.
NOV.
DEC.
JAN.
FEB.
MAR.
APR.
MAY
JUN.
JUL.
1976

TABLE 2. Daily changes in plasma concentrations of thyroid hormones. Samples were taken on June 29, 1976 from the same group of fish used for annual cycle study. Data is presented as mean ± 1 SEM. The range is given in brackets.

Sample time	n	Weight (g)	Standard length (cm)	Sex	T_4 (ng/ml)	T_3 (ng/ml)
0530	4	175±26 (105-227)	22.2±0.8 (19.9-23.7)	3M, 1F	0.5±0.1 (0.3-0.8)	4.7±0.4 (3.7-5.8)
1130	6	199±14 (140-240)	23.9±1.1 (21.9-24.9)	4M, 2F	2.1±0.2 (1.6-2.6)	5.5±0.6 (3.4-6.9)
2030	4	220±22 (174-268)	24.2±0.8 (22.3-25.5)	2M, 2F	2.0±0.7 (0.9-3.8)	6.4±0.7 (4.7-7.8)

The Accelerated Photoperiod Group

Data from three samples of fish taken from the accelerated photoperiod group (APG) are shown in Table 3. The size of the fish were less than those in the annual cycle study. It was noted by the Superintendent of the Raven Station that the fish did not feed appreciably during the first three months in captivity. When the initial sample was taken on April 29, the fish were seen to be blanched and lean. By the end of May, pigmentation had returned to normal. The fish had started to eat by this time as well.

Table 4 compares the gonadal state of fish from the APG with that of corresponding monthly samples from the annual cycle study. It is clear that up to the end of June, the gonads of the fish which had been exposed to an accelerated photoperiod were not different from those of fish exposed to natural daylengths. By June 30, the ova of both groups were beginning the secondary growth phase.

The circulating levels of total T_4 and T_3 of fish in the APG are given in Table 3. It is obvious that both T_3 and T_4 concentrations were low when compared to the corresponding monthly values from the annual cycle study (see Table 1).

Thyroid Hormone-Binding Proteins

Incubation of fish and mammalian plasma for different lengths of time showed that equilibrium was reached by 4 hr at 4^0 and by 2 hr at 20^0. After these times, no appreciable increase in binding occurred.

The relative extent of binding, as indicated by the percent of $*T_3$ or $*T_4$ eluted from Sephadex columns, is shown for three vertebrate species in Table 5. In brook trout, $*T_4$-TBP binding ranges from 1.5-10%

TABLE 3. Thyroid hormone data from accelerated photoperiod experiment. In all samples, n=4. Data is presented as mean ± 1 SEM. The range is given in brackets.

Sample date	Weight (g)	Standard length (cm)	Sex	T_4 (ng/ml) plasma	T_3 (ng/ml) plasma
Apr 29	99±32 (37-189)	18.8±1.5 (15.0-21.7)	4M	1.0±0.2 (0.5-1.4)	1.9±0
May 30	87±5 (74-98)	19.5±0.5 (18.2-20.8)	3M, 1F	0.9±0.5 (0.1-2.0)	2.3±0.6 (0.8-3.5)
Jun 29	88±12 (58-114)	20.1±0.8 (18.4-20.5)	1M, 3F	1.2±0.2 (0.8-1.7)	1.2±0.3 (0.8-1.9)

TABLE 4. The reproductive cycles of *Salvelinus fontinalis* exposed to the natural photoperiod (A) and to an accelerated photoperiod (B). Values are expressed as the mean. Sample size is given in brackets.

Sample date	Ova diameter (mm)		Female GSI		Male GSI	
	A	B	A	B	A	B
April 29	1.20 (2)	- (0)	0.95 (2)	- (0)	0.38 (4)	0.41 (4)
May 30	1.28 (3)	1.10 (1)	1.33 (3)	1.89 (1)	0.47 (3)	0.51 (3)
June 29	1.57 (2)	1.48 (3)	1.78 (2)	1.40 (3)	0.48 (4)	0.59 (1)

and $^{*}T_3$-TBP binding from 20-45%. Thus, in the brook trout, it appears that more T_3 is bound to protein in the plasma than T_4. The mammalian plasma had considerably more bound T_3 and T_4 than did brook trout. In contrast to the teleost, more T_4 was bound to protein than was T_3 in mammalian plasma samples.

Melatonin Assay

The spectrophotofluorometric scan of extracted MT standard (1 µg/ml) is shown in Figure 7. The peak at 550 nm is characteristic of 5-hydroxy- or 5-methoxyindole derivatives in acid (Udenfriend *et al.*, 1955). Table 6 shows that approximately 70% of standard melatonin (1 µg/ml) was recovered when extracted from a solution of 0.1 N HCl containing 0.5% ascorbic acid or when extracted from pineal homogenate. Considering this extraction efficiency, the method was estimated to be capable of detecting 100 ng/pineal in the pooled monthly samples of six pineals.

The writer was not able to detect melatonin in the pooled pineal homogenates of immature rainbow trout, mature rainbow trout, or in any monthly sample of brook trout from the annual cycle study.

TABLE 5. Thyroid hormone protein binding data for three vertebrate species. After incubation with 0.1 ml plasma sample, bound hormone was separated from free hormone by gel chromatography on Sephadex G-25 (fine) equilibrated in 0.1 M sodium phosphate buffer, pH 7.6. The extent of hormone binding to plasma proteins is indicated by the percent of either $^{*}T_3$ or $^{*}T_4$ eluted from the column.

Sample	Percent Eluted $^{*}T_3$	Percent Eluted $^{*}T_4$
Class: *Agnatha*		
Petromyzon marinus		
December	28.4	1.5
	20.0	2.2
April	33.4	5.6
	37.5	7.5
May	32.3	1.6
	44.3	10.0
June	–	7.3
	23.4	3.9
Class: *Mammalia*		
Spermophilus richardsoni		
October-normothermic	80.0	95.8
Homo sapiens		
euthyroid	67.8	74.5

[1] Incubation at 4^{0} for 4 hr.

[2] Incubation at 20^{0} for 2 hr.

Figure 7. Spectrophotofluorometric emission spectrum of melatonin standard (1.0 µg/ml) after selective extraction with [illegible]. The peak of intensity at the wavelength of 550 nm is characteristic of 5-hydroxy- and 5-methoxyindole derivatives in acidic [illegible]. Excitation wavelength was 295 nm. Cell temperature = 20°.

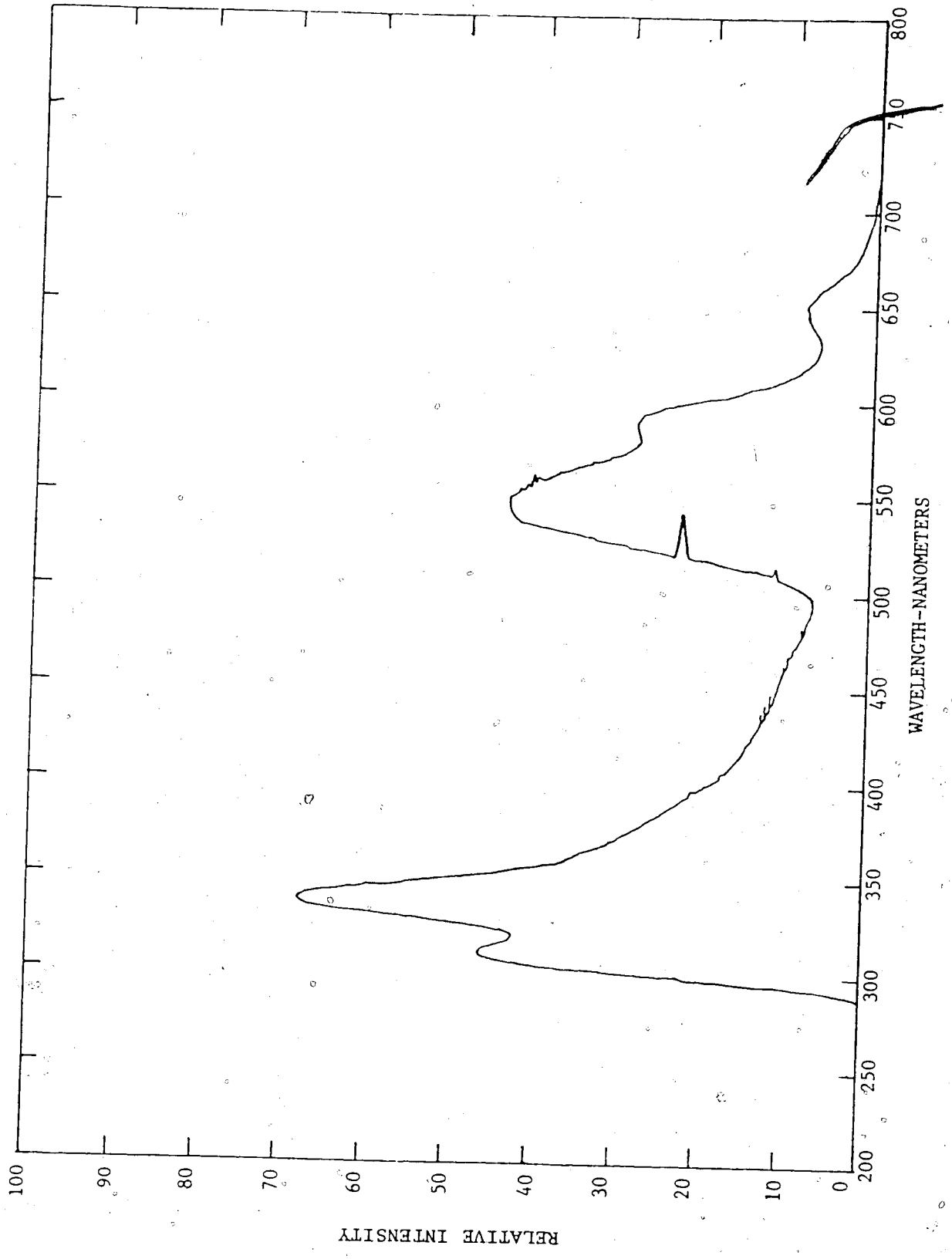

RELATIVE INTENSITY
100
90
80
70
60
50
40
30
20
10
0
200
250
300
350
400
450
500
550
600
650
700
750
800
WAVELENGTH-NANOMETERS

TABLE 6. Efficiency of extraction of melatonin by p-cymene for spectrophotofluorometric analysis (Quay, 1963). Melatonin in solution and melatonin added to pineal homogenate were extracted. Relative intensity (RI) of the extracted sample was compared to that of unextracted standard (1.0 µg/ml). Excitation wavelength was 295 nm. Relative intensity was measured at 550 nm.

Sample and procedure	% Recovery[1,2]
0.1 ml MT standard (1.0 µg/ml), extracted with p-cymene. (4 determinations)	68.5
	75.5
	69.0
	66.0
0.5 ml MT standard (1.0 µg/ml) added to 0.5 ml pineal homogenate (*Salmo gairdneri*). 0.1 ml extracted with p-cymene.	72.3

[1]For the first 4 values,

$$\% \text{ recovery} = \frac{\text{RI of extracted sample}}{\text{RI of unextracted sample}} \times 100$$

[2]For the last value,

$$\% \text{ recovery} = \frac{\text{RI of extracted sample} \times 2}{\text{RI of unextracted sample}} \times 100$$

Discussion

The Existence of Seasonal Cycles

The ability of an organism to maintain a regulated internal environment is regarded as being the most significant phenomenon in the evolution of life. Bernard's concept of homeostasis was an important contribution to the understanding of biological form. However, it is easily misinterpreted as meaning the maintenance of a physiological state that is fixed or confined within narrow limits. Organisms are only partially free from their physical surrounding in that they must depend on their environment for the energy with which they slow the inexorably entropic nature of matter. Thus, the *milieu organique interieur* must be in dynamic equilibrium with the *milieu cosmique ambiant*.

The influence of a seasonally changing environment on man as well as other living organisms has long been appreciated. The cyclic response of animals to seasons (e.g., hibernation, estivation, migration, reproduction, vernal crop planting, etc.) is well known.

In the first half of the twentieth century, a series of observations elucidated the key mechanism by which a major vertebrate control system, the endocrine system, is influenced by the external environment. The pituitary, described at the time as the "master gland", was found to be under partial control by factors originating in the central nervous system, specifically the hypothalamus (Harris, 1937, 1950; Green and Harris, 1947). The recent elucidation of the primary structure of two mammalian hypothalamic hypophysiotropic hormones (Bøler *et al.*, 1969; Burgus *et al.*, 1970, 1972; Matsuo *et al.*, 1971) and the isolation and partial characterization of several others (see Grant and Vale,

1974; Saffran, 1974) have firmly established the neuroendocrine control of the hypophysis.

Temperate zone teleosts, which are exposed to annual changes in such factors as photoperiod, temperature and food availability, display cycles of endocrine activity which allow them to adapt to the seasons (Hoar, 1959). Perhaps the best recognized cycle in fish is reproduction (Hoar, 1969). Seasonal changes in energy utilization (Hoar, 1969) may be controlled by annual cycles of prolactin synthesis and secretion (Sage and de Vlamming, 1975). Furthermore, actions of prolactin may be influenced by cyclical activity of corticosteroids (Meier, 1972). There is also evidence to suggest an annual cycle of growth hormone (GH) production in teleosts (Brett, 1976).

Thus, there is probably an annual pattern of circulating levels in every pituitary hormone and the corresponding target-gland hormone in temperate zone teleosts. The changing concentrations of circulating T_3 and T_4 demonstrated in this study of the brook trout (see Figures 3 and 4) provide clear evidence for an annual cycle of thyroidal activity and responsiveness.

The Concentrations and Nature of Circulating Thyroid Hormones in Fish

The range of values for T_4 reported in the present study show considerable variation over the year (see Table 1). However, even the highest observed value in an individual sample (4.7 ng/ml) is low in comparison to total human plasma T_4 concentrations. Euthyroid human plasma averages approximately 65 ng/ml (Oppenheimer and Surks, 1974) and levels of up to 125 ng/ml T_4 have been reported in pregnant women (Refetoff *et al.*, 1970). Even the lowest levels of T_4 reported in

mammals (Refetoff *et al.*, 1970), 6.0 ng/ml in the horse, *Equus caballus* and 8.0 ng/ml in the monkey, *Macaca mulatta*, are well above the mean T_4 values reported by the writer for the brook trout (see Table 1).

Total plasma T_4 concentrations that have been reported recently for lower vertebrates are shown in Table 7. All values, except those of Brown and Eales (1976) were obtained by competitive protein-binding (CPB) analyses utilizing mammalian TBG as the specific binding protein. The values obtained by this method may be artifically high due to indiscriminate binding of other iodothyronines such as T_3 (Refetoff *et al.*, 1970; Oppenheimer and Surks, 1974). This could be a significant source of error in T_4 estimation in lower vertebrates which may have the same or a higher circulating concentration of T_3 than of T_4 (see below). T_4 titres in lower vertebrates are generally less than those in man. No pattern of T_4 concentration according to phylogenetic development can be discerned based on available data (compare Table 7 with the values reported in Refetoff *et al.*, 1970).

The T_4 values reported in the present study are at the low end of the range of poikilotherm T_4 titres (see Table 7). They compare favorably with other estimations from the same species. However, it is difficult to compare values determined by the writer with those of other investigators who made no mention of the time of year or time of day when their samples were taken. Comparison of the author's data to other accounts is further hindered by the absence of published information on the physiological state of the animal (other than state of nutrition) and on environmental conditions and history.

With the very recent use of RIA for the measurement of T_3, there is little data with which to compare the T_3 values obtained in this

TABLE 7. Total plasma concentrations of T_4 and T_3 reported in lower vertebrates. CPB refers to competitive-protein binding assays utilizing mammalian TBG. RIA refers to the use of an antibody specific to T_4 or T_3. PBI refers to protein-bound iodine.

Species	Assay used	Experimental conditions	T_4 (ng/ml) plasma	T_3 (ng/ml) plasma	Reference
Class: *Cyclostomata*					
Pacific hagfish (*Eptatretus stouti*)	CPB	Kept for 3 wk in aquaria with low salinity. Temp 11-13^0. Fed.	20-30	Not done but PBI was 50-80 µg/ml.	Henderson and Lorscheider, 1975
	CPB	Kept for 8 d, aerated sea water. Temp 8^0. Fed.	34	-	Packard et al., 1976
Atlantic hagfish (*Myxine glutinosa*)	CPB	Kept for 1 wk, recirculating sea water. Temp 6.5^0. Fed.	28-131	-	Henderson, 1976
Pacific lamprey (*Entosphenus tridentatus*)	CPB	Kept several days in covered tank. Temp 8^0. Fed.	5	-	Packard et al., 1976
Class: *Actinopterygii*					
Cutthroat trout (*Salmo irideus*)	CPB	At capture	45	-	Refetoff et al., 1970
Perch (*Catastomus commersoni*)	CPB	At capture	43	Not done but PBI was 88 µg/ml.	ibid

TABLE 7 (continued).

Species	Assay used	Experimental conditions	T_4 (ng/ml) plasma	T_3 (ng/ml) plasma	Reference
Class: *Actinopterygii* (cont'd)					
Brook trout (*Salvelinus fontinalis*)	CPB	At local hatchery	1.5-3.0	-	Higgs and Eales, 1973
	CPB	At laboratory. Temp 12-13^0. Fed.	3.0-33.6	-	ibid
	CPB	At laboratory. Temp 12-13^0. Starved.	0-9.5	-	ibid
Channel catfish (*Ictalurus punctatus*)	CPB	At capture	27.0	-	ibid
Goldeye (*Hiodon alosoides*)	CPB	At capture	0.7	-	ibid
Rainbow trout (*Salmo gairdneri*)	RIA	At laboratory. Temp 11-13^0. Fed.	0.9-6.1	0.5-6.2	Brown and Eales, 1976
Class: *Amphibia*					
Leopard frog (*Rana pipiens* complex)	CPB	Running sink water	17	-	Packard et al., 1976

study (see Tables 1 and 7). The values of T_3 are similar to those given by Brown and Eales (1976) from pooled plasma samples of rainbow trout (see Table 7). As with T_4, a detailed comparison is precluded by lack of information on the fish which were examined by Brown and Eales. Although there is disagreement as to normal T_3 concentrations in humans, it is estimated to be 1-3 ng/ml, or approximately 2% of total plasma T_4 concentrations (van Middlesworth, 1974). Like T_4, levels of T_3 measured by CPB (1.5-2.5 ng/ml) were higher than levels measured by RIA (0.7-1.9 ng/ml) (see "Normal Laboratory Values", New England Journal of Medicine, 1974). The levels of both T_3 and T_4 in the brook trout reported in the present study (measured by RIA) and by Higgs and Eales (1973; measured by CPB) fall within the range of T_3 values in man.

In the brook trout, it is striking that T_3 levels were consistently higher than those of T_4 (see Figure 5). Although estimates of both circulating iodothyronines in fish are few, at least one study agrees with the author's observations. Jacoby and Hickman (1966) estimated values of circulating T_3 and T_4 to be 13 and 11 ng/ml, respectively, in rainbow trout, *Salmo gairdneri*. From analysis of thyroid tissue, the same authors found that the intrathyroidal quantity of T_4 was six times greater than that of T_3. Assuming proportional secretion rates of iodothyronines by the thyroid (Pitt-Rivers and Rall, 1961), the circulating values of T_3 and T_4 in the brook trout suggest the following:

(1) There is considerable peripheral deiodination of T_4, possibly to T_3. Estimates of up to 42% of T_4-to-T_3 conversion have been obtained in mammals (Oppenheimer and Surks, 1974). Higgs (1974) found the rate of $*T_4$ deiodination to be greater than that of $*T_3$ in the

brook trout. In addition, he reported the appearance of a large quantity of $*T_3$ after injection of $*T_4$. It is not known if defodination is an enzymatic or non-enzymatic process in fish (Higgs, 1974).

(2) There is a high rate of T_4 excretion. There is considerable biliary excretion of both thyroid hormones in the brook trout (Eales, 1970; Eales *et al.*, 1971). $*T_4$ removal seems to be more rapid than $*T_3$ (Eales *et al.*, 1971; Higgs, 1974).

(3) The potential for T_4 removal by cells is similar to that of T_3. In man, the distribution volume of T_3 is approximately 50 ml/100 g and T_4 is approximately 14 ml/100 g (Larsen, 1972). In the brook trout, the distribution volume is about 15 ml for both hormones (Higgs, 1974).

The rate of clearance of thyroid hormones from the blood is a function of thyroid hormone-TBG binding (Ingbar and Woeber, 1974). The proportion of free T_4 in fish is equal to or less than that of free T_3 (Eales *et al.*, 1971; Falkner and Eales, 1973). However, methods such as equilibrium dialysis and protein precipitation may measure loose association of hormones with plasma proteins as well as strong binding.

Qualitative comparisons of thyroid hormone-TBP binding may afford a better understanding of phylogenetic differences in iodothyronine economy. Such comparisons have suggested that binding in lower vertebrates is weaker than in mammals. Farer *et al.* (1962) reported smeared electrophoretic patterns near the anode of T_3 and T_4 with plasma of lower vertebrates as opposed to distinct bands of these hormones which were discretely associated with protein peaks of TBG, albumin and prealbumin in mammalian samples. The use of starch gel support, which weakly competes for thyroid hormones, caused dissociation of T_3 and T_4 from proteins in lower vertebrates (Farer *et al.*, 1962). Falkner and

Eales (1973) also reported dissociation of T_3 and T_4 from proteins using starch gel electrophoresis in plasma samples of *Salvelinus fontinalis*.

The data reported here on T_3- and T_4-TBP binding (see Table 5) agrees with the earlier findings in fish that show a weak association of thyroid hormones with plasma proteins. In human and ground squirrel sera, thyroid hormone binding is stronger to TBP than to the Sephadex G-25 matrix. Also, in mammals, the percent of bound T_4 appears to be higher than that of bound T_3. In the brook trout, T_3 and T_4 binding to TBP appears to be consistently weaker than binding to the Sephadex gel. In contrast to mammalian samples, the binding of T_3 to TBP appears to be stronger than the binding of T_4 to TBP.

In lower vertebrates, which lack the specific, high affinity TBG of mammals (Farer *et al.*, 1962; Tanabe *et al.*, 1969), methods which measure a loose association of T_3 and T_4 with plasma proteins may not provide data which can be interpreted in terms of their physiological value. In the brook trout, a larger proportion of T_3 was found to be unbound than of T_4, as measured by protein precipitation. Yet the biliary excretion of $*T_4$ was found to be greater than that of $*T_3$ (Eales *et al.*, 1971). The binding data from the present study show T_4-TBP binding to be weaker than T_3-TBP binding. This suggests that the disposal rate of T_4 may be greater than that for T_3 and may account for the low levels of circulating T_4 and for a T_3/T_4 ratio that is greater than unity.

Another explanation of lower circulating levels of T_4 than of T_3 is simply that less T_4 is being produced by the thyroid. This could be a consequence of low environmental iodine; it is more economical to use three iodine molecules per iodothyronine than it is to use four (Studer

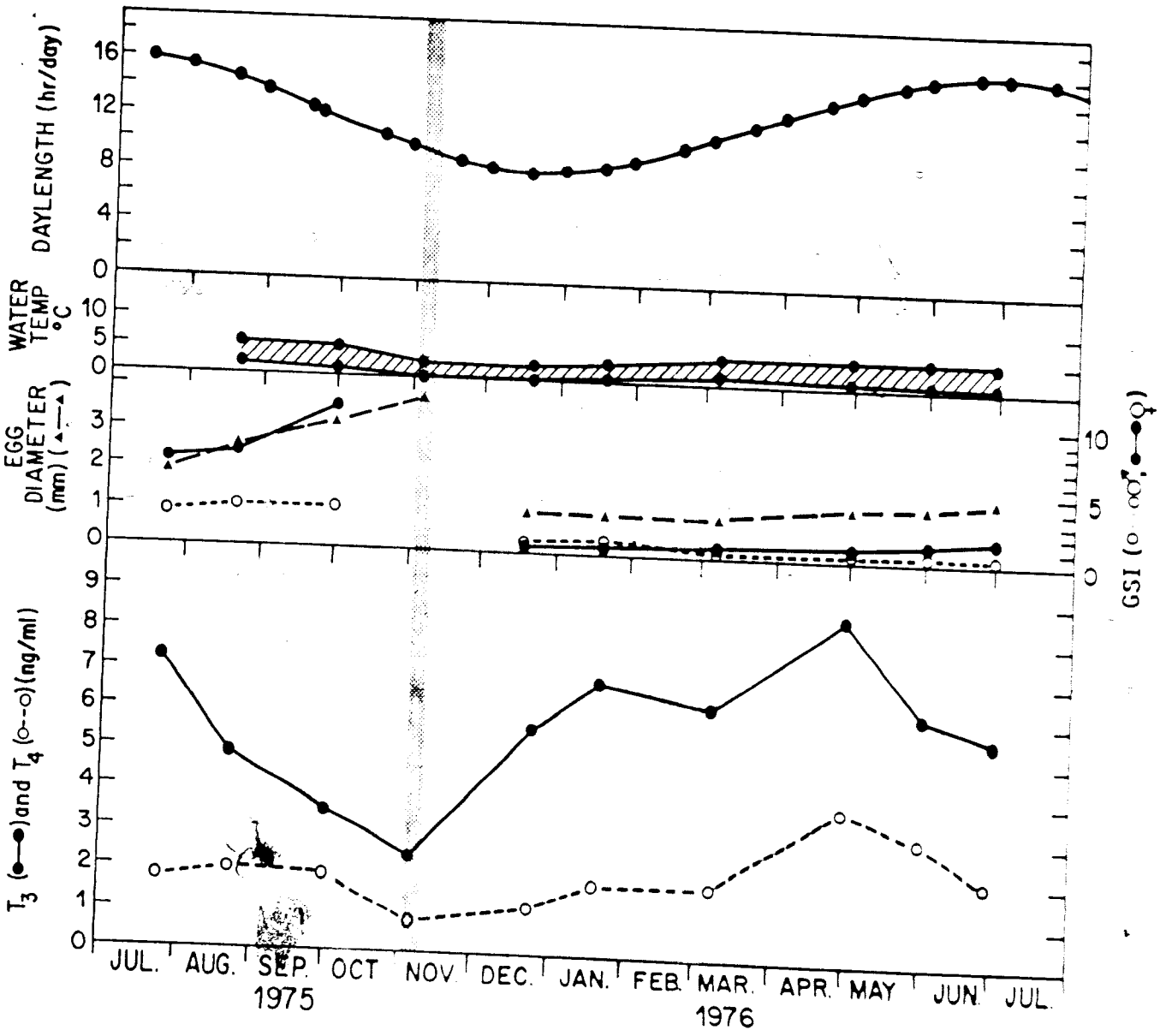
et al., 1974). However, this is not supported by analysis of thyroid tissue (Jacoby and Hickman, 1966). More insight into the iodine-iodothyronine economy could be provided by RIA determination of the intrafollicular T_3 and T_4 concentrations in the brook trout.

If one considers the free levels of T_4 (approximately 0.1-0.5 ng/ml) and the free levels of T_3 (approximately 0.01 ng/ml) in mammals (van Middlesworth, 1974), teleosts appear to have more thyroid hormone readily available to the cellular binding proteins. The implications of this are not clear. In general, it seems that fine controls in thyroid hormone action mediation such as low levels of free, circulating hormone available to high affinity cellular receptors specific to T_3 (Oppenheimer and Surks, 1975) and large stores of circulating T_4-TBG and T_3-TBG to buffer the demand of the cells (van Middlesworth, 1974) are absent in present day teleosts. Instead, teleosts may represent a stage in thyroid evolution in which T_4 and T_3 have similar intrinsic biological activities.

Possible Causal Factors in the Cycles of Circulating T_3 and T_4

The annual profiles of T_3, T_4, GSI, ova diameter, temperature and photoperiod are summarized in Figure 8. The changing levels of T_3 and T_4 are clear evidence for an annual cycle of circulating concentrations in both thyroid hormones. The timing of these cycles is probably influenced by seasonal changes in the environment. Examination of Figure 8 suggests that a simple relation between plasma levels of thyroid hormones and either photoperiod or temperature does not exist. Although T_3 and T_4 reach their lowest concentrations at a time of decreasing photoperiod, both show increasing levels when daylength is still

Figure 8. Summary of data from annual cycle study showing daylength for latitude of 50 n, maximum and minimum monthly temperatures, the reproductive cycle (egg diameter, male GSI and female GSI) and mean circulating levels of total T_3 and T_4. The vertical bar denotes time of spawning.


DAYLENGTH (hr/day)
16
12
8
4
0
WATER TEMP °C
10
5
0
EGG DIAMETER (mm) (▲—▲)
3
2
1
0
GSI (○ ♂, ● ♀)
10
5
0
T3 (●—●) and T4 (○--○) (ng/ml)
9
8
7
6
5
4
3
2
1
0
JUL.
AUG.
SEP.
1975
OCT
NOV
DEC.
JAN.
FEB.
MAR.
APR.
MAY
JUN.
JUL.
1976

diminishing. Similarly, the spring peak occurs at a time of increasing photoperiod, yet the levels of both hormones begin to decrease before the summer solstice. In this study, where fish were held in a pond with a swift water current, temperature remained continuously low, varying no more than 4^0 during a month and no more than 5^0 over the entire year (Figure 8). It is highly unlikely that these minor variations in temperature account for the fluctuations in hormone levels.

The levels of T_3 and T_4 may have been responding to physiological changes within the fish. As the endocrine system controls long-term seasonally sensitive processes of vertebrate physiology, the changing levels of thyroid hormones suggest a functional relation between the thyroid axis and other endocrine axes. These may be occurring centrally (hypothalamus and hypophysis) or peripherally.

The most obvious indication from the present study of the thyroid's complicity with other endocrine-controlled events is seen in the circulating levels of both hormones in November (Figure 8). The fact that lowest levels of T_3 and T_4 occur at the time of spawning suggest some involvement of the thyroid hormones in the control of reproduction. In exploring the nature of thyroid-reproduction association, one must consider the meaning of an alteration in circulating hormone concentration. The circulating level of a hormone is the net result of rate of secretion and rate of disposal. One interpretation of declining hormone levels is a lower rate of synthesis and secretion without alteration of the rate of utilization or metabolic clearance.

The cyclicity of gonadal maturation in fish is controlled primarily by gonadotropin (GTH) release (see review, de Vlamming, 1974). Circulating levels of [illegible] increase in male and female brook trout and salmon

(Salmo salar) in the late stages of spermiogenesis and oogenesis, reaching their highest values during the spawning period (Crim *et al.*, 1975). Gonadal steroid levels gradually increase during development and differentiation of ovarian and testicular tissues (Barr, 1968; Lofts, 1968). In brook trout, it is thought that the decreasing day-lengths of summer and autumn serve as a stimulus to the rapid phase of gametogenesis, possibly augmenting GTH release (Henderson, 1963).

T_3 levels begin to decline at the time of increasing gonadal activity (July; Figure 8). Decreasing photoperiod may stimulate release of TIF and thus decrease the circulating levels of TSH. This could cause a preferential inhibition of thyroid T_3 secretion (Taurog, 1974), lowering the ratio of circulating T_3 to T_4 (see Figure 5).

In a recent review, M. Fontaine (1976) commented on the possibility that the thyroid influences GTH secretion in fish. Thus, low levels of T_3 may sensitize the gonadotropes in brook trout to decreasing day-length. Sage and Bromage (1970b) found evidence of a negative feedback between T_4 and gonadotrope activity in the guppy. LaRochelle and Freeman (1974) have shown that thyroidectomized rats display a greater response of LH and FSH secretion to ovariectomy. Thus, in the brook trout, the GTH cells may be released from an inhibitory feedback by thyroid hormones during the summer months. The decline of T_4 levels in the month prior to spawning (Figure 8) may have a similar sensitizing role. The combined effects of low levels of both thyroid hormones could contribute to the surge in circulating GTH observed during the month prior to spawning by Crim *et al.* (1975).

In addition to decreasing photoperiod, low levels of T_3 and T_4 prior to spawning could be due to an inhibitory action on the thyroid

axis by rising levels of gonadal steroids. In the guppy, Sage and Bromage (1970b) obtained evidence for a negative feedback of gonadal hormones on the activity of pituitary thyrotropes.

A second interpretation of altered thyroid hormone levels is a change in the disposal rate of T_3 and T_4 with a constant rate to synthesis and secretion. It is possible that T_3 and T_4 binding to cellular proteins may irreversibly remove the hormones from the circulation (Oppenheimer and Surks, 1975). Barrington and Matty (1954) speculated that the thyroid was involved directly with gonadal development. Increased utilization of thyroid hormone (in the autumn) is further suggested by the fact that the standard metabolic rate of brook trout is highest during the spawning period (Beamish, 1964). Normal thyroid secretion is a prerequisite to proper gonadal function, particularly in females, in both fish (Ball, 1960) and higher vertebrates (Bray and Jacobs, 1974). If thyroid hormones, especially T_3, were involved in the control of the rapid phase of gametogenesis (July to November), then increased utilization might account for the reduced levels of thyroid hormones. This implies that the feedback mechanisms are unable to ensure constant blood levels of thyroid hormones in the face of inhibition by decreasing photoperiod and increasing gonadal steroids.

The two interpretations of decreasing thyroid hormone levels are not necessarily mutually exclusive. Thyroidal stimulation of gonadal development may occur in summer, before high titres of GTH or steroids are secreted. Subsequently, decreasing levels of T_3 and T_4 may allow increased GTH secretion and thus, increased steroidogenesis. In the final phase of maturation the possible gonadotropic role of T_3 and T_4 may be replaced by gonadal steroids. Thus, thyroid hormones may be

gonadotropic at the level of the gonads and still exert an indirect antigonatropic effect through an inhibitory influence on GTH secretion.

In considering the low levels of T_3 and T_4 during the spawning period, attention should also be given to the availability of iodine for thyroid hormone synthesis. The iodine content of the pond water was very low (<1.0 µg/l) in comparison to the concentrations reported for many freshwater bodies (1-6 µg/l; Livingstone, 1963). The principle source of iodine was that contained in the food given to the fish (approximately 3 mg iodine/kg food). Feeding, and thus, dietary intake of iodine is decreased prior to the breeding period. This could have the effect of lowering the rate of iodothyronine synthesis and blood levels of T_3 add T_4. Iodine is accumulated by the ovaries (Leloup and Fontaine, 1960). Thus, the rapid growth of ova prior to spawning might diminish the supply of iodine to the thyroid (Sage, 1973). If this reduction was significant, one would find differences between male and female trout at or near the time of spawning. No such differences were found in fish sampled at any point in the year.

Another possible endocrine-thyroid association in the brook trout is that with growth hormone. Although thyroid hormones are known to have profound effects on growth in higher vertebrates (Ingbar and Woeber, 1974), their role in teleost growth is far from clear. On the basis of available data, Gorbman (1969) concluded that in fish, the thyroid hormones may have only a permissive effect.

In freshwater teleosts, growth is stimulated by long or increasing daylengths. In laboratory studies, an increased growth rate is usually not observed until six to eight weeks after application of a stimulatory light regime (Brett, 1976). The circulating titres of both T_3 and T_4

reach their maximum levels in the brook trout in mid-spring when daylength is increasing (Figure 8). It is possible that the high levels may be a response to increasing daylength that is associated with an enhanced growth rate typically seen in spring and summer months. In addition to being directly involved in the anabolic processes of growth, thyroid hormones may have an indirect stimulatory influence on growth by promoting the rate of secretion of pituitary growth hormone (Sage, 1967).

The possible bases of thyroid-gonadal and thyroid-growth relations discussed above are speculative. The possibility exists that the link between the thyroid and reproduction or between the thyroid and growth may be correlative rather than causal, with all three activities being regulated by seasonal changes in environmental factors. The possibility of the existence of a role of the thyroid in reproduction and growth needs to be resolved by further work.

Daily Variations in Thyroid Hormone Levels

Although limited, the data reported in Table 2 suggest the existence of a diurnal cycle in the circulating levels of T_3 and T_4 in brook trout. The general trend appears to be an increase in levels of T_3 and T_4 during daylight hours. As in the annual cycle study, light seems to exert a stimulatory effect on thyroid secretion. Daily patterns of feeding and locomotion are also likely to be involved in changes in circulating titres of the thyroid hormones.

The existence of diurnal rhythms in blood levels of hormones in vertebrates is well known (see Weitzman *et al.*, 1975; M. Fontaine, 1976). The observations in brook trout point to the need for a more detailed study. More importantly, they emphasize the need for investigators

to exercise care in the design, interpretation and reporting of their experiments. In the annual cycle study reported here, all blood samples were collected within the same 90 minute period (1030-1200 hr).

Accelerated Photoperiod Group

Exposure of brook trout to an accelerated light regime had no effect on the rate of gonadal development (see Table 4). The experiment was discontinued in August, 1976. The negative result is likely due to the fact that the fish were in their first reproductive cycle. While an accelerated light regime can hasten the time of functional maturity in adult brook trout which are in their second or succeeding reproductive cycle, such a regime is without effect when applied to fish in their first cycle (Henderson, 1963).

The data from the APG provide a clear example of dependency of thyroid metabolism on the physiological state of the animal. The fish in the APG were wild-captured and confinement in the experimental tank unboubtedly served as a major stressor. Non-specific trauma has profound effects on the thyroid axis (LaRochelle and Freeman, 1974). Lack of feeding was no doubt a manifestation of trauma. The levels of T_3 and T_4 in the three samples were subnormal (Table 3) when compared to the levels of the corresponding monthly values from the annual cycle study (Table 1). This agrees with Higgs and Eales (1973) who found generally lower T_4 values in starved than in fed brook trout (see Table 7). Higgs (1974) also found a general depression of thyroid metabolism during starvation. This study re-emphasizes the necessity of considering experimental conditions before comparing data or forming conclusions (Barrington *et al.*, 1961).

Melatonin

The extraction efficiency reported here (Table 6) compares favorably with that obtained by Quay (1963; 73% recovery). The detection limit of the assay (100 ng) was well below the levels reported in salmon pineals by Fenwick (1970a; 180 ng/pineal in mature fish).

In the light of previous studies, it is surprising that no 5-MAT was detected in brook or rainbow trout pineals. Recently, we were advised by J. C. Fenwick (pers. comm.) that before assaying 5-MAT in rainbow trout, the fish had been held in total darkness for several days prior to sampling. It seems likely that the discrepancy between Fenwick's observations and those reported here is due to the fact that the fish used in my study were exposed to natural cycles of daylength. Investigation of the pineal demands the development of more sensitive techniques such as RIA for the measurement of normally-occurring concentrations of 5-MAT.

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Appendix I

As mentioned in the text, the Mallinckrodt kit T_3 RIA was used initially. In this assay, antibody and plasma sample are added to a vial containing $*T_3$ and 8-anilonapthalene sulfonate (ANS), an inhibitor of T_3-TBG binding. After 90 minutes of incubation, the unbound T_3 ($*T_3$ and T_3) is removed by adding an anion-exchange resin strip for 60 minutes.

If no antibody is added, then all T_3 should be unbound, and thus removed during the resin strip incubation. However, in vials with rainbow trout plasma and no antibody, up to 40% of $*T_3$ remained after the resin strip incubation.

Two approaches were taken in an attempt to reduce this false "bound" value.

(1) Increasing the potential of the anion-exchange resin strip to remove bound hormone.

(2) Removing any non-specific binding agents (e.g., protein or lipid) by chloroform-methanol extraction.

To achieve a better separation of bound from free fraction, the incubation time of the fractions with the resin strip was increased from the recommended time of 60 min to 120 min in 15 min intervals. Also, two resin strips were placed in vials in order to increase the total surface area of the ion-exchange resin. Both of these attempts to increase the capacity of the separation step had no significant effect on the false "bound" radioactivity. Thus, it was concluded that other agents existed whose binding was not inhibited by ANS.

Next, chloroform-methanol extraction was tried as it not only denatures proteins but also provides a non-polar phase which removes some

of the lipid. The extraction method was one used earlier by the writer at the Massachusetts General Hospital. The procedure was as follows:

(1) 0.5 ml plasma sample, 0.5 ml H_2O, 2.5 ml $CHCl_3$ and 5.0 ml CH_3OH were added to a 60 ml separatory funnel. The contents were mixed, depressurized and incubated at room temperature for 30 min.

(2) 2.5 ml $CHCl_3$ was added, the contents mixed and depressurized, and the two phases allowed 30 min at 4^0 to separate.

(3) The methanolic phase was transferred to a 15 ml centrifuge tube and the denatured protein was precipitated by centrifugation at 3000 x G for 30 min.

(4) The supernatant was transferred to a 10 ml test tube. The pellet was washed once with 1 ml $CHCl_3$ and this was added to the supernatant. The tubes were then placed in a 30^0 water bath and $CHCl_3$ was evaporated by introducing a gentle stream of nitrogen into each tube.

(5) The residue was resuspended in 0.5 ml distilled water. 100 µl aliquots were assayed from each sample.

Reproducibility was tested by extracting several 0.5 ml aliquots of pooled plasma from three rainbow trout. Percent recovery was tested by comparing extractions of a known amount of standard with unextracted standards.

The method reduced non-specific binding from 40% to 7%. However, percent recovery, and therefore, reproducibility was not consistent. Also, by this time, the first plasma samples from the annual cycle study (see text) had been taken and it was clear that an assay requiring lower sample volumes would be needed. Finally, the Mallinckrodt assay was

abandoned with the availability of a more reliable and easier extraction method, the alkaline Sephadex assay (Brown and Eales, 1976; see above).

Appendix II

The protocol of the radioimmunoassays for T_3 and T_4 is given in detail by Brown and Eales (1976). The Sephadex columns are equilibrated and stored with 0.1 N NaOH. All reagents and columns must be at room temperature. The columns are drained, the bottom capped and the plasma sample (50-200 µl) and tracer (labelled hormone) (100 µl) are applied to the column. The column is swirled gently, the cap removed and the column allowed to drain. Lipids and denatured protein are washed from the column with 3 ml buffer. The columns are then placed over 10x75 mm culture tubes and 1.0 ml of the antiserum solution applied to the column. The column is covered and incubated for 90 min. The hormone-antibody complex is eluted from the column with 2 ml buffer. The tubes are capped with parafilm and counted for 10 min or 10,000 CPM.

The buffer used for the T_4 RIA was 0.075 M barbitol buffer, pH 7.4. The buffer used for the T_3 RIA was 0.1 M sodium phosphate (dibasic) with 0.03 M EDTA, pH 7.4.