THE UNIVERSITY OF MANITOBA

PLASMA L-THYROXINE AND TRIIODO-L-THYRONINE IN IMMATURE RAINBOW TROUT, *Salmo gairdneri* Richardson; THEIR MEASUREMENT AND FACTORS INFLUENCING THEIR LEVELS

by

Scott Brown

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"PLASMA L-THYROXINE AND TRIIODO-L-THYRONINE IN IMMATURE RAINBOW TROUT, SALMO GAIRDNERI RICHARDSON; THEIR MEASUREMENT AND FACTORS INFLUENCING THEIR LEVELS"

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Radioimmunoassay (RIA) procedures suitable for measuring L-thyroxine (T_4) and 3, 5, 3'-triiodo-L-thyronine (T_3) levels in 0.05 to 0.30 ml of rainbow trout plasma are described. Mean detection limits were 12.5 ng/100 ml (T_4 RIA) and 9.5 ng/100 ml (T_3 RIA); mean indices of precision were 0.056 (T_4 RIA) and 0.042 (T_3 RIA). Both "within" and "between" assay reproducibilities were satisfactory. Storage of plasma for 10 days at -20°C did not alter measurements of plasma T_4 or T_3.

Plasma T_4 and T_3 ranged from 10-600 ng/100 ml in the rainbow trout. Generally, T_4 was about 200 ng/100 ml while T_3 was about 120 ng/100 ml. Starved, immature trout showed no significant diurnal or sex-related differences in plasma T_4 or T_3. A positive correlation existed between body weight and the plasma hormone level; both plasma T_4 and T_3 increased by about three percent per gram body weight. MS 222 anaesthesia had little effect on plasma T_4 or T_3. MS 222 anaesthesia followed by cardiac injection of trout doubled plasma T_4 and reduced plasma T_3 by 50 percent. Plasma thyroid hormones were sometimes lower in starved fish. EWOS trout food contained 0.87 ng/g T_4 and 0.49 ng/g T_3 and these levels were felt not to influence plasma T_4 or T_3 levels.

In conclusion, the RIA techniques described were capable of detecting physiological differences in plasma T_3 and T_4. Stress factors and starvation were important variables influencing circulating thyroid hormones of rainbow trout.

INTRODUCTION

Plasma levels of L-thyroxine (T_4) in teleost fish are usually below 500 ng/100 ml (ng %) rendering assay difficult (Higgs and Eales 1973). Plasma levels of 3, 5, 3'-triiodo-L-thyronine (T_3) have not been routinely quantified in teleosts.

Accurate measurements of plasma thyroid hormones are required as a basis for understanding several important variables of thyroid function. They are required, for example, to assess hormone degradation rates in kinetic studies. They are also required for estimation of free hormone levels in serum or plasma (Burke and Eastman 1974). Sensitive techniques for measuring thyroid hormones can be further applied to tissue extracts (Nejad et al. 1975). With increased emphasis on the importance of T_4 to T_3 conversion to activate the hormone (Sterling 1970; Pittman et al. 1971; Sterling et al. 1973), tissue hormone levels and rates of production may provide important insights into thyroid hormone metabolism.

Current methods for plasma T_4 or T_3 measurement include competitive protein-binding assays (CPB) (Higgs and Eales 1973; Henderson and Lorscheider 1975), gas-liquid chromatography (GLC) (Osborn and Simpson 1972) and radioimmunoassay (RIA) (Alexander and Jennings 1974b; Sterling and Milch 1974). Considering sensitivity, specificity, speed and economy, RIA is the method of choice (Burke and Eastman 1974). The first objective of this study was to devise RIA methods suitable for measuring T_4 and T_3 in teleost plasma.

The second objective was to investigate certain commonly-encountered

variables that might influence plasma T_4 and T_3 levels. The variables chosen were diurnal variation, stress of anaesthesia or injection, and nutritional state.

Rainbow trout were chosen because they were readily available and have been used previously in thyroid as well as other metabolic studies.

LITERATURE SURVEY

1. Measurement of thyroid hormones in fish blood.

Until recently estimates of thyroid hormones in teleost plasma were based on protein-bound iodine (PBI) determinations (Leloup and Fontaine 1960; Hickman 1962; La Roche et al. 1965; Jacoby and Hickman 1966). The inadequacies of using PBI for fish plasma have been pointed out by Jacoby and Hickman (1966) and Higgs and Eales (1973). The PBI fraction can be influenced by inorganic iodide in the plasma and also does not distinguish between T_4 and T_3.

The PBI measurement was superseded by the more sensitive and reliable CPB techniques using thyroxine-binding globulin to measure T_4 (Refetoff et al. 1970; Higgs and Eales 1973). While CPB assays were an improvement, they lack the sensitivity and precision necessary for measuring the low levels of T_4 found in fish plasma (Higgs and Eales 1973). Also, CPB assays cannot be easily used to determine plasma T_3 values (Burke and Eastman 1974).

Few attempts have been made to quantify circulating T_3 in teleosts. Jacoby and Hickman (1966) identified the proportions of T_4 and T_3 occurring in trout plasma. By combining this with PBI measurements they estimated plasma T_4 and T_3. This technique is laborious and requires numerous fish, therefore, it cannot be applied as a routine laboratory procedure. Osborn and Simpson (1972) used GLC to measure plasma T_4 and T_3 in the plaice. The values must be treated with reserve due to the possibility of artificial deiodination of T_4 to T_3 during the GLC procedure (Nihei et al. 1971).

RIA techniques offer the sensitivity, specificity and precision necessary to measure accurately low levels of hormones in plasma (Skelley et al. 1973; Zettner 1973; Burke and Eastman 1974). The principles of CPB assays and RIA are basically the same, except that antisera to T_4 or T_3 replace thyroxine-binding globulin as the specific binding agent. Equilibrium RIA and CPB assays rely on the partial saturation of the specific binding agent by the substance undergoing testing. The test substance competes with a radioactive, chemically similar or identical indicator substance for the available binding sites on the binding agent (Zettner 1973). The more unlabelled substance present the less labelled substance becomes bound to the binding agent. By holding the concentration of labelled substance and binding agent constant while increasing the concentration of the unlabelled substances, one can obtain a progressive response which is plotted as a standard curve. Quantification of the unknown sample is possible by interpolation from the curve.

Miniature Sephadex columns are useful in measuring serum T_4 and T_3 in human serum (Bauer et al. 1970; Alexander and Jennings 1974b). When serum is applied to Sephadex columns, equilibrated with 0.1N NaOH, T_4 and T_3 are released from their binding proteins and are adsorbed onto the Sephadex. The serum proteins are then eluted from the columns with barbital buffer at pH 8.6. The lower pH permits subsequent binding of T_4 or T_3 by specific binding substances that are added to the columns. A second rinse removes the binding substance-bound T_4 or T_3, and allows determination of plasma T_4 or T_3. The Tetralute procedure used by Higgs and Eales (1973) employed Sephadex columns. Although their method

lacked sensitivity, use of Sephadex columns along with antisera to T_4 or T_3 and high-specific-activity radioactive T_4 or T_3 may provide the necessary increase in sensitivity.

RIA and CPB kits are available from a variety of sources. These kits are designed to measure circulating T_4 or T_3 in humans. In man, T_4 levels are approximately 8-10 µg % (Ingbar and Woebar 1974), while in fish T_4 levels are generally less than 0.5 µg % (Higgs and Eales 1973). Therefore, kits are not sensitive enough to permit accurate assessment of plasma T_4 in teleosts. Kits for plasma T_3 may be suitable to measure teleost plasma T_3. The levels are similar (Brown, this thesis). However, analysis of T_4 and T_3 in plasma using the commercial kits is costly.

2. Diurnal variation in thyroid function.

Daily fluctuations of thyroid function have been extensively studied in mammals and birds, often with conflicting results. Nicoloff et al. (1970), Van Cauter et al. (1974), Azukizawa et al. (1975), Fukuda et al. (1975a) and Fukuda and Greer (1975) gave evidence for diurnal variations in plasma thyroid stimulating hormone (TSH) for man and rats. Other studies failed to show diurnal TSH variations in man (Odell et al. 1967; Webster et al. 1972). There are also reports of cyclical patterns in thyroid hormone metabolism (Walfish et al. 1961) and thyroid gland activity (Walser et al. 1963; Woods et al. 1966; Nicoloff et al. 1970; Newcomer 1974; Krebietke 1975b). Whether or not diurnal rhythms exist for plasma T_4 and T_3 is uncertain in mammals. The available studies are outlined in Table 1.

So far, no study of diurnal variations in thyroid function has been

Table 1. Summary of various studies on daily rhythms in plasma levels of thyroid hormones.

Source	Animal	Variable Measured	Results
Lennon and Mixner (1957)	Cow	PBI	No variation at 8-hr intervals.
Margolese and Golub (1957)	Man	PBI	Significant day to day variations appear cyclical rather than random.
Tingley et al. (1958)	Man	PBI	No variation between 0700, 1300 and 1700 hr.
Schatz and Volpe (1959)	Man	PBI	No variation at 0800, 1330, 1730 and 0300 hr.
Walfish et al. (1961)	Man	PBI	No significant day-to-day variation and states not as sensitive as disappearance rate.
Walser et al. (1962)	Man	PBI	Significant fluctuation maximum 0030 hr and 0730 hr with gradual decrease rest of day.
Auerbach (1963)	Man	PBI	Marked increase in PBI 0730-1230 hr decreasing to minimum 2200-2400 hr. The fluctuation parallels adrenal activity.
Yousef and Johnson (1966)	Cow	PBI	No diurnal variations when sampled at 3 hr intervals.
Lemarchand-Béraud and Vanotti (1969)	Man	PBI FT_4	No variation in PBI but sampling every 4 hours showed a circadian variation of free-T_4 inversely related to TSH concentration.

...cont'd

Table 1 (continued)

De Costre et al. (1971)	Man	T_4 FT_4	Significant fluctuation for total T_4 not related to food, or adrenal cortical function; more related to postural changes. No change in FT_4 levels.
Sadovsky and Bensadouin (1971)	Chicken	Iodo-hormones by chroma-tography (T_4 and T_3)	Diurnal variation for plasma hormones but values at least 2X higher than PBI values reported. Technique has been questioned by Newcomer (1974).
Ban (1972)	Man	T_4 FT_4	No significant fluctuation when sampled every 3 hr.
Surks et al. (1972)	Man	T_4 T_3	Both hormones measured at 3-hr intervals and no significant variation.
Vernikos-Danellis et al. (1972)	Man	T_4 T_3 Index	Fluctuations in both hormones with peaks at 0730, however low amplitude fluctuations disappear with bed rest.
Grandesso et al. (1974)	Man	T_4 T_3 Index	Significant fluctuation higher at 0800 and lowest at 2400 hrs for T_4, T_3 index drops at 1200 hr recovering by 2000 hrs.
Mühlen et al. (1974)	Rat	T_3	Levels decreased from 0800 to 2000 hr increasing after 2000 hr, somewhat similar to TSH which was highest at 0400 hr and lowest at 2000 hr.
Newcomer (1974)	Chicken	T_4 T_3	When sampling at 3-hr intervals both hormones are statistically observed to fluctuate in diurnal sinusoidal wave forms the crests not coinciding in time.
O'Connor et al. (1974)	Man	T_4	Sampling at 20-min intervals over 24 hr, indicated acute short duration T4 fluctuation in subjects confined to bed.

...cont'd

Table 1 (continued)

Azukizawa et al. (1975)	Rat	T_4 T_3	Both hormones fluctuated periodically but pattern was inconsistent and did not correlate with rhythmic TSH levels.
Balsam et al. (1975)	Man	T_4 T_3	Sampling at 2 hr intervals T_4 levels varied significantly, highest at 0900 hr, lowest at 1500-1700 hr. T_3 highest at 0900 hr and lowest at 1700-1900 hr. Total plasma protein did not vary.
Fukuda et al. (1975a)	Rat	T_4 T_3	Sampled at 3 hr intervals. No daily rhythm for T_3 or T_4 levels, however, TSH levels fluctuated rhythmically and independently of corticosteroids.
Horth et al. 1975)	Man	T_4	Significant fluctuations highest at 1500-1800 hr and lowest at 0300-0600 hr.
Johns et al. (1975)	Man	T_4 FT_4 Index	Sampled 0715, 1300, and 2230 hr. Significant diurnal variations in T_4 concentration. FT_4 index varied little.
Krebietke (1975a)	Chicken	PBI FT_4 Index	Maximum level were at 0300 and 2100 hr and minimum levels were at 1200 hrs for both PBI and "free" hormone. Diurnal changes in albumin and hematocrit were not concordant.
Nye et al. (1976)	Man	T_4	Sampled at 2 hr intervals between 0800-1600 hrs and 1200-0400 hr. The variation observed was slightly higher than interassay variation but no pattern.

published for fish. Several authors have demonstrated daily fluctuations for prolactin and glucocorticoids in teleost plasma (Garcia and Meier 1973; Leatherland and McKeown 1973; Fryer 1975; Spieler and Meier 1976). In view of daily variations in these other hormones in teleosts and possible cyclical thyroid function in other animals, a study of daily changes in thyroid hormone levels in fish plasma may be a valuable aid to the design of future experiments.

3. Sex and thyroid function

Limited data exist on sex-related variations of thyroid function in fish. Differences between sexes have been reported for thyroid gland protein-bound iodine and thyroid hormone content (Matty 1960; Matty and Thornburn 1970), epithelial cell height (Pickford and Atz 1957), and iodide binding to plasma proteins (Huang and Hickman 1968). Other authors have noted no difference between sexes for T_4 response to TSH (Ortman and Billig 1966; Chan and Eales 1976), epithelial cell height (Chavin 1956), plasma protein-bound iodine (Matty 1960) or conversion ratio (Hoar and Eales 1963). Since sex-related differences in plasma TSH, T_4 and T_3 levels and T_4 turnover rates exist in mammals (Gregerman 1963; Rapp and Pyun 1974; Fukuda et al. 1975a), it was felt that evaluation of sex differences in teleost plasma thyroid hormones would be of value.

4. Body weight and thyroid function

Matty (1960) and Matty and Thornburn (1970) reported a significant correlation between body weight and thyroidal PBI and thyroid levels in

the Bermuda parrot fish. Chan and Eales (1976) noted increased thyroid response to TSH in larger brook trout with the relationship being almost linear. For man, Bray et al. (1976) reported a body weight correlation for T_3 and related the observed differences to caloric intake and metabolic rate. It therefore, seems possible that body weight may be related to plasma T_4 or T_3 in teleosts.

5. Anaesthesia and thyroid function

Anaesthesia causes altered thyroid function in man and experimental animals. Several authors report lower plasma TSH following anaesthesia (Ducommun et al. 1966; Wilber and Utiger 1967; Melander 1970; Ohtake and Bray 1975). In some cases the decreases in plasma TSH were followed by decreases in radioactive iodine secretion from the thyroid (Melander 1970; Ohtake and Bray 1975). Anaesthesia had no effect on pituitary TSH release which was stimulated by exogenous thyrotropin-releasing hormone (TRH) (Koch et al. 1972; Ohtake and Bray 1975). This lack of effect indicates that anaesthetic agents are not directly inhibiting pituitary TSH release. Therefore, the lower plasma TSH reported above must be due to either greater thyroid hormone feed-back on the pituitary and brain or depressed central nervous function and subsequent lower release of endogenous TRH.

Several studies indicate rapid increases in plasma PBI and T_4 following anaesthesia (Fore et al. 1966; Oyama et al. 1969a,b; Harland et al. 1974). Elevations in plasma T_4 and free T_4 begin immediately following anaesthesia and are not attributable to surgical trauma (Oyama et al. 1969a; Brandt et al. 1976).

Little evidence exists linking teleost thyroid function and anaesthesia. Chan and Eales (1976) reported no effect of MS 222 treatment on plasma T_4 response to TSH. Anaesthesia can cause increased plasma corticosteroid concentrations in teleosts (Fagerlund 1967; Wedenmeyer 1969). However Wedenmeyer (1970) noted that MS 222 neutralized to pH 7 or benzocaine anaesthesia had no effect on fish corticosteroid levels. In the light of differences recorded in mammalian systems and possible changes in fish corticosteroid function, the effects of anaesthesia on teleost thyroid function appear worthy of investigation.

6. Surgical stress and thyroid function.

Surgical stress or intraperitoneal injection decreased plasma TSH in rats (Ducommun et al. 1966; Fukuda et al. 1975a). Laparotomy and intravenous saline injection reduced plasma radioactive iodide and PBI. This was accomplished by lower release of radioactive iodine from the thyroid gland in rodents (Brown-Grant et al. 1954; Brown-Grant and Pethes 1960; Melander 1970). On the other hand in man, a burst in thyroid gland activity followed some time after even moderate surgery (Harland et al. 1972; Kirby et al. 1973). Serum T_3 fell with elective surgery, while serum T_4 remained the same or rose slightly (Bermudez et al. 1975; Burr et al. 1975a, b; Brandt et al. 1976). Serum free-hormone levels or free hormone indices are usually elevated, indicating diminished binding by plasma proteins. This would result in lower concentrations of plasma thyroid hormones.

Few records are available concerning fish thyroid function and surgical stress. Vehicle injection had no effect on plasma response to TSH (Chan and Eales 1976). Henderson (1976) reported decreased

plasma T_4 in the hagfish and Lewis and Dodd (1976) found lower plasma T_3 in the shark after capture. Osborn and Simpson (1972) found lower levels of both thyroid hormones in the plaice after capture stress. A post-operative increase in plasma PB ^{131}I was reported for catfish (Collicutt and Eales 1974). Serial blood sampling, restraint or injection all cause increased corticosteroid concentration in teleost plasma (Hane et al. 1966; Speiler 1974; Fryer 1975). Differences in plasma T_4 or T_3 may also be detectable, but have not been investigated so far.

7. Nutritional state and thyroid function.

The influence of nutritional state on thyroid function has received much consideration in mammals. During prolonged food deprivation plasma TSH levels (D'Angelo 1951; Vinik 1975) as well as TRH secretion (Shambaugh and Wilber 1974) are reduced. When starvation was less severe no difference in TSH was noted (Ingenbleek and Beckers 1975; Merimee and Fineberg 1976). Deprivation of food usually causes thyroid gland hypoplasia and subsequent lower thyroidal activity in mammals, including man (Rabinovitch 1929; Stephens 1940; Pipes et al. 1960; Grossie and Turner 1962; Stirling 1962; Talwalker and Gaitonde 1974). Decreased metabolic clearance of T_4 and lower peripheral conversion of T_4 to T_3 also occur in starved mammals (Nathanielsz 1969; 1970; Ingbar and Galton 1975).

Plasma PBI and T_4 either show little change or a slight decrease in starved mammals (Schatz et al. 1967; Deb et al. 1973; Portnay et al. 1974; Chopra and Smith 1975; Vagenakis et al. 1975). Plasma T_3 in

mammals always decreases during starvation (Portnay et al. 1974; Rastogi et al. 1974; Chopra et al. 1975a; Vagenakis et al. 1975).

In fish, the influence of nutritional state on thyroid function has not received as much attention. Vilter (1944) first suggested that decreased thyroid function occurred in eels during periods of starvation. Since then other authors have reported decreased epithelial cell height and general gland atrophy during starvation (Ortman and Billig 1966; McBride 1967). Fontaine and Fontaine (1956) and Bonnet (1970) found decreased thyroidal uptake of radioactive iodide in rainbow trout and mullet that had been starved or had important dietary constituents removed. Fed brook trout and rainbow trout had increased metabolic clearance rates, T_4 deiodination, and biliary excretion when compared to starved fish (Eales and Sinclair 1974; Higgs 1974).

Plasma hormone levels have not been thoroughly investigated in fed and starved fish. Osborn and Simpson (1972) reported decreased iodothyronine levels after starvation in the plaice. Higgs (1974) sometimes found increased plasma T_4 in fed fish. Similar results have been suggested in cyclostomata (Henderson and Lorscheider 1975, Packard et al. 1976).

Considering the effects of food deprivation on mammals and similar trends observed in fish, further study on the influence of nutritional state and thyroid function in teleosts appears necessary.

Feeding thyroid powder has a stimulatory effect upon growth in length and weight of yearling rainbow trout (Barrington et al. 1961). Since dietary hormone may influence fish growth, larger amounts of thyroid hormones in the diet might influence plasma hormone levels.

Magwood and Heroux (1968) found significant amounts of T_4 (580 ng/gm) in a manufactured rat diet. Similarly, significant amounts of T_4 or T_3 might be found in manufactured fish diets. Thus, before studying the influence of feeding on plasma thyroid hormones, the amount of hormone in the diet should be determined.

MATERIALS AND METHODS

1. Fish maintenance.

One-year-old rainbow trout, Salmo gairdneri Richardson, were obtained from the Provincial Trout Hatchery, West Hawk Lake, Manitoba and from the Federal Fish Hatchery, Balmoral, Manitoba. Whitefish, Coregonus clupeaformis (Mitchill), were netted several years previously from Lyons Lake, Manitoba.

Several hundred stock trout were held in 2.3-kl tanks with flowing, aerated, dechlorinated Winnipeg City water. They were fed a diet of one to 1.5% (percentage wet body weight per day) Ewos trout pellets (Astra Chemicals Ltd., Mississauga, Ontario).

Experimental fish were held in 125-ℓ fiberglass tanks. The water temperature ranged, seasonally, from 10.5 to 13.5°C. During any one experiment, the water temperature varied at most by one degree C. The tanks were covered with translucent plastic and the photoperiod was adjusted to 12 hours light and 12 hours darkness (light 0600-1800). Water flow and aeration were adjusted to be as uniform as possible between tanks. Further details of holding conditions and rations are given in the protocol for individual experiments.

2. Plasma collection.

Fish were netted five or six at a time and placed into aerated, isothermal, 20-l, covered tanks of water. Individual fish were anaesthetized by immersion in MS 222 (Kent Laboratories, Vancouver; 0.06 g/l), blotted dry, and weighed to the nearest 0.1 g. Blood was withdrawn from the caudal vessels with a prehaparinized one-millilitre tuber-

culin syringe (25 gauge, 5/8"-needle). The samples were expelled into 1.5-ml plastic centrifuge tubes and centrifuged at 15000 g for three to five minutes (International Centrifuge model MB). The plasma was aspirated with a Pasteur pipet and stored at -20°C in two-millilitre plastic beakers covered with Parafilm. All plasma samples except control samples for the RIA procedures, were analysed for thyroid hormones within 10 days of blood collection.

3. T_4 and T_3 RIA

i) Reagents. Barbital buffer (pH 8.6; 75 mmol/l) was prepared by dissolving 15.6 g of sodium barbital in 900 ml of distilled, deionized water. The pH was then adjusted to 8.6 with 6N HCl (2.0-2.5 ml) and diluted to one litre (Seligson and Seligson 1972).

^{125}I-T_4 (T_4*) and ^{125}I-T_3 (T_3*) phenolically labelled and with initial specific activities of approximately 725 and 500 mCi/mg respectively, were purchased from Industrial Nuclear, St. Louis, Mo. in 50% aqueous propylene glycol. (These levels of specific activity are required for sensitivity in the tests.) The T_4* or T_3* was diluted with 0.1N NaOH to provide stock solutions such that 0.1 ml generated 3500-4500 cpm in a gamma well-detector of approximately 50% efficiency. These solutions were stored at -20°C and were useable for a maximum of four weeks.

Standard stock solutions of T_3 and T_4 (10 µg% as free acid) were made by dissolving the anhydrous sodium salt of T_3 (Sigma) or T_4 tablets (Eltroxin, sodium L-thyroxine pentahydrate) in 0.1N NaOH. These stock solutions were stored at four degrees and remained stable at least four months. As needed, working standards of 0, 50, 100, 200, 400, 600,

or 800 ng% were prepared by further dilution with 0.1N NaOH.

Lyophilized rabbit antisera to T_4-human serum albumin (T_4 antibody) or to T_3-human serum albumin (T_3 antibody) were purchased from K and T Biological Services Ltd., Edmonton, Alberta. The supplier's specifications indicate that the T_4 antibody exhibits less than 1% cross reaction with T_3 and triiodothyroacetic acid, 1.3% with triiodothyropropionic acid, 10% tetraiodothyroacetic acid, and 25% with tetraiodo-desamino-thyronine. The T_3 antibody shows less than 1% cross reaction with T_4, 2.8% with tetraiodothyroacetic acid, 20% with triiodothyroacetic acid, and 80% with triiodothyropropionic acid.

The raw (undiluted) T_4 antiserum was diluted 1:7000, and the raw T_3 antiserum was diluted 1:22000 with barbital buffer. (These dilutions may vary somewhat with the strength and source of antisera used. If these factors are unknown the antisera should be diluted until 50-60% binding is achieved with the "0 ng" standard.) Reconstituted antibody was stable in barbital buffer about four weeks at four degrees C. Addition of sodium azide (1 gm/l) increased the storage life.

The particular Sephadex columns used in this study were those supplied with the Tetralute competitive binding kit (Higgs and Eales 1973). Each column was prepared from 0.45 g of G-25 (Fine) Sephadex. Column preparation and maintenance have been described elsewhere (Bauer et al. 1970; Seligson and Seligson 1972; Alexander and Jennings 1974a). Screening of columns greatly aided reproducibility. This was achieved by following the normal assay procedure, except that 0.1 ml of the 100 ng% standard of either T_4 or T_3 was added to each column. Only those columns whose bound fraction fell within a range of 100 cpm of each other were used. After an assay the columns were regenerated by eluting with five millilitres

of distilled, deionized water (pH 6.4), two millilitres of human plasma (diluted 1:20 in barbital buffer), and finally 10 ml of water. The columns were then eluted with 10 ml of 0.1N NaOH and stored capped at room temperature with one or two millilitres of 0.1N NaOH above the column surface. Bastomsky et al. (1976) recommended fresh columns be prepared every three months.

ii) Radiation counting. Samples of three millilitres (T_3 RIA) or four millilitres (T_4 RIA) were counted for 10,000 counts per min (cpm) or 10 min in a Nuclear Chicago Automatic Gamma System containing a two-inch (DS 202) NAI crystal.

iii) Assay procedure.

1. Allow all reagents and columns to come to room temperature before use.

2. Allow the columns to drain and cap the bottoms.

3. Add 0.1 ml of T_3 or T_4 standard or 0.1 ml of plasma on to each column. Duplicate standards should be run at each concentration.

4. Add 0.1 ml of T_4* or T_3* solution to each column.

5. Pipet 0.1 ml of T_4* or T_3* in duplicate into counting tubes, add 3.9 ml (T_4 RIA) or 2.9 ml (T_3 RIA) of barbital buffer to each tube. Cap and set aside as the total counts reference (TCR).

6. Swirl each column and allow to drain to waste.

7. After tracer and standards or plasma have completely drained into each column, add four millitres (T_4 RIA) or three millilitres (T_3 RIA) of barbital buffer to each column, and allow eluate to run to waste. (These volumes will remove iodide contamination and serum proteins from the columns).

8. After the buffer has drained through the column, position each column over an empty counting tube and add one millilitre of T_3- or T_4- antibody reagent to each column. When many columns are used "time-sequence" the antibody addition.

9. Allow the column to equilibrate for 90 minutes. They should be covered to prevent drying out.

10. Following equilibration, add three millilitres (T_4 RIA) or two millilitres (T_3 RIA) of barbital buffer to the columns in the same "time-sequence as stage '8'. This results in volumes of four millilitres (T_4 RIA) or three millilitres (T_3 RIA) in the counting tubes. (All bound antibody is removed with the first two millilitres of buffer. The free fraction remains with the Sephadex columns.)

11. Plot T_4 or T_3 standard curves of antibody-bound cpm ('y' axis) against hormone concentration (ng %) ('x' axis). Determine unknown hormone concentration by interpolation.

12. The standard curves should be determined with each assay, with a control serum to insure adequate quality control.

13. If, in any group of fish analysed, hormone concentration was zero, the detection limit of that particular assay was used. This was felt to be more representative, since it would be unlikely that the hormone concentration would be zero.

14. If the eluate is collected from sample columns in stage '7' this will permit estimation of T_4* or T_3* recovery of those particular samples by the Sephadex. If this eluate is collected from any two columns comprising the standard curve this will permit determination of the radioiodide contamination of the T_4* or T_3* used. In the case of T_3 2% should be subtracted from the value obtained to correct for the loss of approximately 2% of the T_3 in the eluate.

4. Evaluation of RIA method.

The ratio of free radiohormone (retained on Sephadex) to antibody-bound radiohormone (F/B) was plotted against hormone concentration (ng %) on 'x' axis. In both assays the plot happened to approximate a linear relationship to which regression analysis could be applied, facilitating statistical evaluation of assays. In order to calculate F/B it was necessary to subtract the radioiodide contamination (stage '7') from TCR (stage '5') and obtain the true quantity of T_4* or T_3* added to each column. From this value and the bound radiohormone obtained by elution, the free radiohormone (retained on the Sephadex) could be calculated.

The index of precision[a] (Midgley et al. 1969) and the detection limit, or sensitivity[b] (Ekins 1974) were determined from the regression of each assay.

The coefficient of variation[c] (CV %) (Watson and Lees 1973) was used to determine "within" and "between" assay reproducibility.

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a $$\text{Index of Precision} = \frac{\text{Standard deviation of regression slope}}{\text{regression slope}}$$

b $$\text{Detection Limit} = \frac{\text{Standard deviation of 'y' intercept}}{\text{regression slope}}$$

c $$\text{Coefficient of Variation} = \frac{\text{Standard deviation of mean hormone concentration}}{\text{mean hormone concentration}} \times 100$$

5. T_4/T_3

The ratio of the concentration of the two hormones (T_4/T_3) is sometimes reported. A constant ratio in the face of changing hormone concentrations may indicate non-specific changes in blood volume or changes in binding of T_4 and T_3 by plasma proteins (Balsam et al. 1975). On the other hand, changing T_4/T_3 along with changing hormone concentrations might indicate alterations in hormone metabolism. Although highly speculative, some insights might be gained by calculating T_4/T_3. In this study T_4/T_3 was calculated as the ratio of T_4 concentration (n mole/l) to T_3 concentration (n mole/l). Molar concentrations are used because it is unfair to compare weight-based concentrations. T_3 contains one fewer iodide atom and weighs only 84% as much as T_4.

6. Analysis of food for T_4 and T_3.

The following procedure was adapted from Magwood and Heroux (1968) for extraction of thyroid hormones from Ewos trout pellets.

1. Trout pellets were ground with a mortar and pestle until mechanically uniform.

2. The ground mixture (1 g) was homogenized with 37.5 ml of ethanolic ammonia (99:1, v/v) in a Sorval Ominimixer for 15 min).

3. The homogenizer chamber and propellor were washed with 12.5 ml of ethanolic ammonia. The mixture was placed on a magnetic stirrer and five-millilitre amounts were transferred into centrifuge tubes (Sorval 18 x 112 mm). The container was well rinsed with ethanolic ammonia. Equal volumes of the rinse were added to each tube.

4. In order to test for recovery, T_4* or T_3* (10,000 cpm) was added in 100 µl of ethanolic ammonia to each of duplicate tubes, which

were stoppered and placed on a shaker at room temperature for 20 min. At the same time 100 µl of T_4* or T_3* was added to duplicate counting tubes and brought to one millilitre with 0.1N NaOH. The counting tubes were capped and set aside.

5. Each tube of food and ethanolic-ammonia mixture was centrifuged for 10 min at 6000 rpm (Sorval General Laboratory Centrifuge). The supernates from the six unlabelled tubes were pooled and divided equally into two test tubes (12.5 x 175 mm). The supernates from all labelled tubes were collected in 15-ml conical tubes.

6. Two millilitres of ethanolic ammonia were added to each centrifuge tube and the tubes Vortex mixed for 30 sec. The mixtures were again centrifuged and the supernates added to the respective fractions. This wash step was repeated.

7. The supernate tubes were placed under jets of air in a fume hood and were warmed to 37°C in a water bath. The tubes were evaporated to dryness and reconstituted in one millilitre of 0.1N NaOH. The material in the labelled tubes was counted and compared to the total count tubes to obtain the extraction efficiencies. Samples (100-400 µl) from the two unlabelled tubes were analysed by RIA for thyroid hormones.

7. Statistical Analysis.

Wherever two means were compared by t-tests, two-tailed F tests were used to test for homogeneity of variance. Barlett's X^2 test was used to test homogeneity of variance when more than two means were considered. If variances were heterogeneous a $\log_{10}$ transform was used to reduce variance of untransformed data. Taylor's power law (Southwood 1971) usually confirmed the validity of the $\log_{10}$ transformation.

Back-transformed data with 95% confidence intervals are reported in figures and tables.

One-way analysis of variance (ANOVA) was used to compare means between groups if one factor was considered. Two-way ANOVA was used when two factors were considered. If the F-tests from the ANOVA techniques proved significant, Newman-Keuls test to locate differences between more than two means was used. Correlation and regression analyses were used to establish any dependence of plasma hormone levels on body weight and to compare T_3 RIA with another assay. Covariance analysis was used to compare hormone levels between sexes. All statistical techniques employed are outlined in Sokal and Rohlf (1969) or Snedecor and Cochran (1971).

EXPERIMENTAL PROTOCOL

1. Diurnal variation in plasma T_4 and T_3.

Rainbow trout (Idaho stock; initial mean weight 24.8g, SEM 0.7g) were randomly divided into five groups of 20 to 24 fish. The fish were fed a 1.5% ration at 1230 hr (midpoint of light period) for 14 days. Then they were starved for three days to inhibit any endogenous hormone variations associated with feeding. The fish were sampled in groups of 10-12 at 0500, 0700, 1030, 1700, and 2230 hr on day four of starvation. Sampling continued at 0200, 1030, 1400, 1900, and 2230 hr on day five.

Caution was taken to avoid disturbing the fish in the controlled-environment room. A dark-room lamp and a small flashlight were used to take samples in darkness. For both T_4 and T_3, plasma samples were too numerous (over 100) to analyse in a single RIA run. Samples were randomly allocated to two RIA runs. This distributed any interassay variation throughout all groups.

2. Anaesthesia - immediate effects on plasma T_4 and T_3.

Twenty-three rainbow trout (Montana stock; mean weight 35.1g, SEM 1.95) were held for eight days on a 1.5% ration. The fish were then starved for three days and sampled on day four with alternate fish being bled without the aid of anaesthesia.

3. Effects of anaesthesia and stress of cardiac injection on plasma T_4 and T_3

Trout (Montana stock; mean weight 27.2g, SEM 1.3g) were randomly divided into four groups of 20-22 fish. They were fed a 1.5% ration

for nine days and then starved three days prior to injection. Alternate fish from three groups were heart injected (Higgs 1974). One group was injected with 20 µl of physiological saline (Hickman's saline; NaCl 6.42, KCl 0.15, $CaCl_2$ 0.22, $MgSO_4$ 0.12, $NaHCO_3$ 0.084, NaH_2PO_4 0.06 g/l). The other was sham injected with no saline being administered. A one-millilitre Hamilton syringe with a PB-600-1 repeating dispenser (30-gauge, 1/2"-needle) was used. All injected fish were lightly anaesthetized (MS 222, 0.06 g/l) prior to cardiac puncture. Control fish were also divided into two groups. Ten fish were anaesthetized and handled similarly (removed from the water 10 sec) to injected fish; the remaining 10 fish were left untouched in the tank. To distinguish groups in each tank, a small portion of the caudal fin was clipped. The handled control fish and the saline-injected fish were clipped. The injected and sham-injected fish were bled two, six, and 12 hr post injection (pi). These times were chosen since they are commonly used sampling times for kinetic studies (Higgs 1974). Control fish were bled at two hours after the beginning of the experiment.

4. Effect of seven days' starvation on plasma T_4 and T_3.

Twenty-four Idaho trout were divided into two groups consisting of 11 and 13 fish (A, mean weight 41.4g, SEM 1.5g; B, mean weight 40.3g, SEM 1.1g) and fed a three percent ration for four days. On day five the fish in group B were starved while those in group A were maintained on continued ration. Seven days later all fish were weighed and bled. Fed fish were sampled between 1330 and 1430 hr, one hour after feeding.

5. Effect of time of starvation or feeding on plasma T_4 and T_3

Idaho trout were grouped into four groups of 15 fish (A, mean weight 45.8g, SEM 2.3g; B, mean weight 43.6g, SEM 2.7g; C, mean weight 47.6g, SEM 3.0g; D, mean weight 44.4g, SEM 3.0g). The fish were held for 10 days on a three percent ration. Then groups C and D were starved while A and B were maintained on three percent ration. After three, seven and 14 days of starvation five fish were sampled from each group. The fish were bled one hour after feeding.

RESULTS

1. Investigation of variables associated with a functional T_4 and T_3 RIA

Several variables involved in obtaining a functional assay design were investigated. These included volume of plasma added to each column, volume of 0.1N NaOH used as the tracer vehicle, separation of plasma iodide contamination and non-specific elution of tracer, incubation time and buffer pH, and separation of bound antibody.

Routinely, 0.1 ml of trout plasma was applied to each column. Figure 1 shows that up to 0.4 ml of trout plasma may be used on each test column for both T_4 or T_3 before significant interference with the column recovery occurs.

The volume of 0.1N NaOH used as the tracer vehicle in stage '4' was studied. For the T_3 RIA, the volume of NaOH should not exceed 0.1 ml. In agreement with Alexander and Jennings (1974b), larger volumes will cause loss of T_3* from the column in stage '7' elution (Fig. 2). For the T_4 RIA, no larger increase of T_4* loss from the column could be found with up to 0.55 ml of NaOH (Fig. 2).

Removal of iodide contamination and non-specific elution are shown in Figure 3. Figure 3A illustrates that 99.9% of iodide contamination will be removed from 0.1 ml of plasma by three millilitres of barbital buffer. Figures 3B and 3C show the amount of labelled iodide removed from T_4* or T_3*. Note that approximately 2% non-specific elution of labelled T_3 occurs, therefore it would be desirable to keep

Figure 1. The percent radioactivity from T_4*(●) or T_3*(o) washed off the Sephadex columns by the initial buffer wash (3 ml T_3 RIA; 4 ml T_4 RIA) in relation to the volume of rainbow trout plasma applied to each column. Each point is the mean of duplicates.

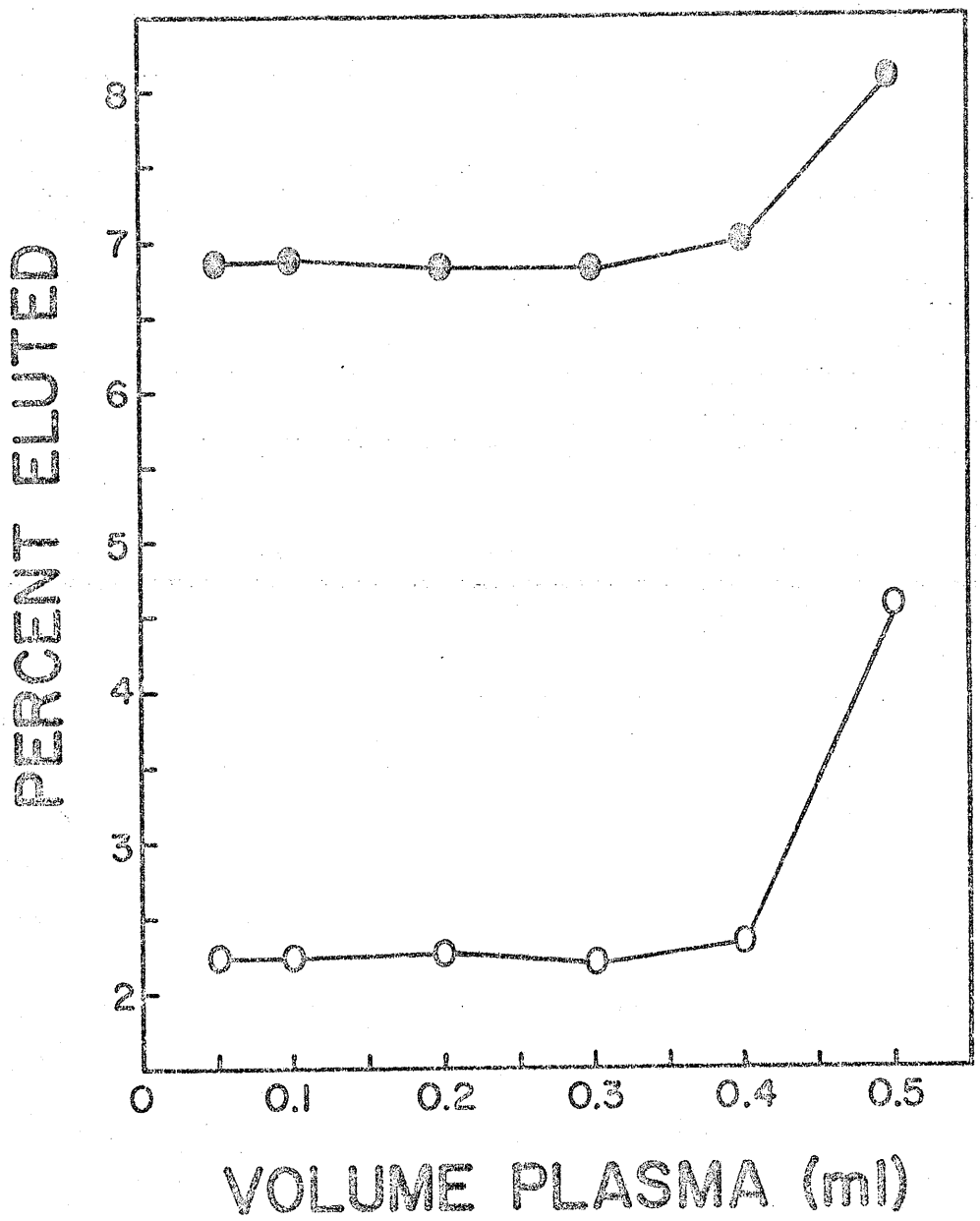

PERCENT ELUTED
8
7
6
5
4
3
2
0
0.1
0.2
0.3
0.4
0.5
VOLUME PLASMA (ml)

Figure 2. Relationship between volume of 0.1N NaOH used to dilute the T_4*(●) or T_3*(o) applied to the column and the percentage of the applied radioactivity eluted with buffer (3 ml for T_3 RIA; 4 ml for T_4 RIA). Each point is a mean of duplicate determinations.

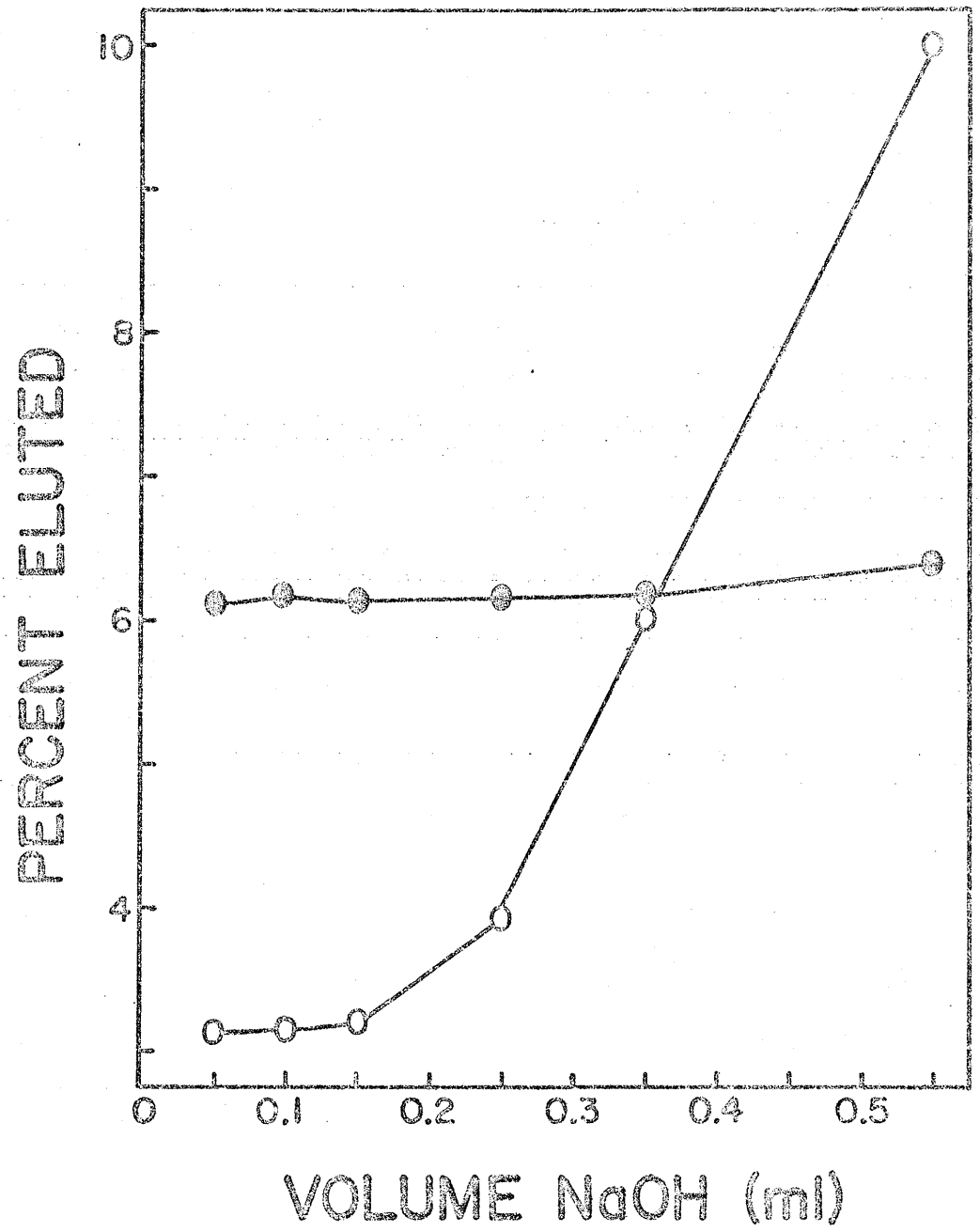
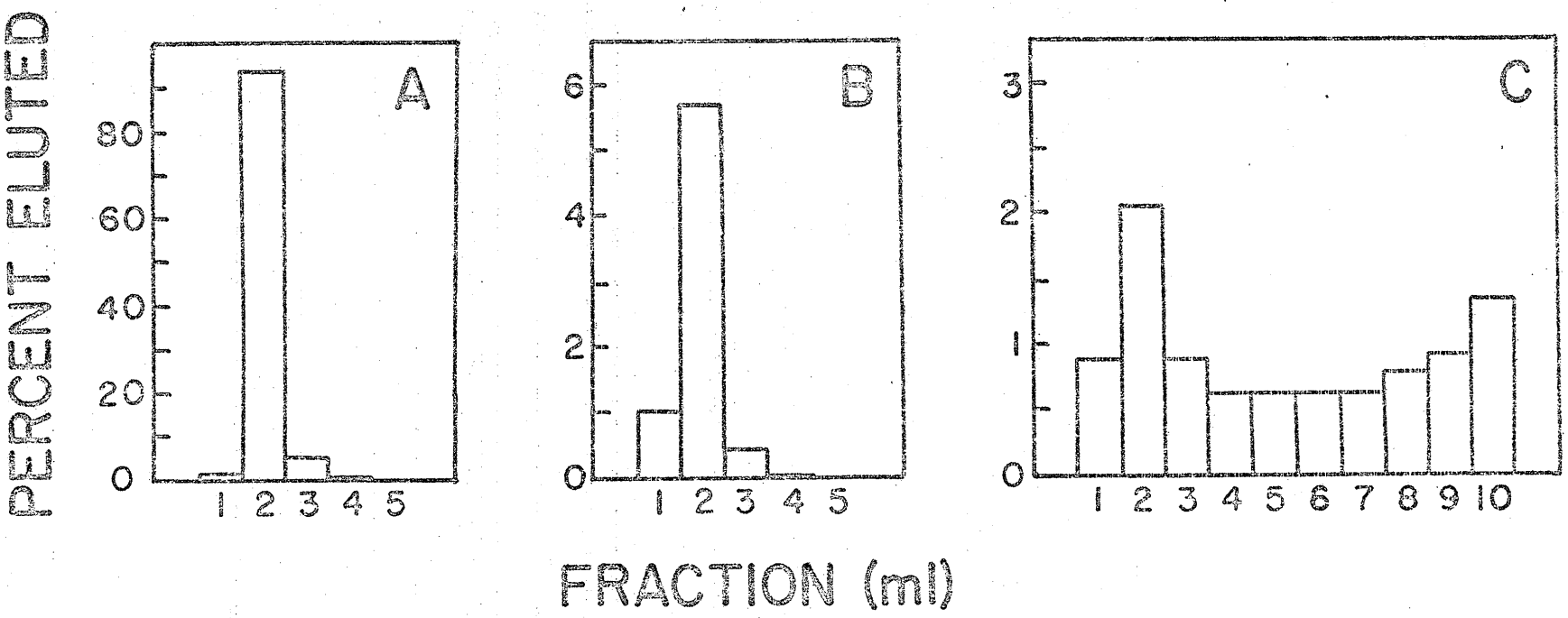

PERCENT ELUTED
10
8
6
4
0
0.1
0.2
0.3
0.4
0.5
VOLUME NaOH (ml)

Figure 3. Percentage of the radioactivity applied to Sephadex columns in 0.1 ml of 0.1N NaOH which is removed with successive 1-ml eluates of buffer. Values are means of duplicates. Similar values were obtained when plasma was used in conjunction with the 0.1N NaOH and tracer indicating close correspondence in elution of radioactivity by 'standard' and 'sample' columns.

A shows that 99.9% of applied $^{125}I^-$ was eluted in the first 3 ml.

B shows that 7.1% of radioactivity applied as T_4* was eluted in the first 3 ml but none thereafter. The $^{125}I^-$ contamination of the T_4* was 7.1% determined independently by electrophoresis, indicating negligible T_4* elution with 10 ml of buffer.

C shows that 4.0% of radioactivity applied as T_3* was eluted in the first 3 ml and significant quantities in subsequent fractions. The $^{125}I^-$ determined independently was 2.2%, indicating 1.8% loss of T_3* in the first 3 ml and even more in later fractions.


PERCENT ELUTED
A
B
C
0
20
40
60
80
0
2
4
6
0
2
3
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5 6 7 8 9 10
FRACTION (ml)

buffer volumes used in the T_3 RIA to a minimum.

Figure 4 shows the percentage of T_4* or T_3* bound to the antibody in relation to incubation time (stage '9'). Beyond 90 minutes, there was negligible slope to either curve. Figure 4 also demonstrates that greater binding occurs with barbital buffer at pH 8.6.

In stage '10' all bound antibody is separated from the free (Sephadex-bound) fraction with the first two millilitres of buffer (Fig. 5). Similar elution patterns were obtained when the columns were charged with 0.1 - 0.4 ml of trout serum. This indicates that the serum proteins in stage '7' are also removed in two millilitres of buffer. Stage '7' requires at least three millilitres to remove iodide contamination .

2. Evaluation of T_4 and T_3 RIA.

Typical T_4 and T_3 standard curves are shown in Figure 6. Figure 7 shows the same data expressed as F/B versus hormone concentration. Table 2 illustrates the regression characteristics of 16 T_4 standard curves and 16 T_3 standard curves. For the T_4 RIA the mean index of precision was 0.056 with a range of 0.043 to 0.090; for T_3 RIA the mean index was 0.042 with a range of 0.021 to 0.085 (Table 2). The mean detection limit for T_4 RIA was 12.5 with a range of 8.8 to 22.0 ng %; for T_3 RIA it was 9.5 with a range of 4.9 to 16.3 ng %.

The "within-assay" reproducibility (Table 3) was evaluated by making eight to 20 single determinations during single assays on samples drawn from rainbow trout plasma pools. The coefficient of variation ranged from 5.1 to 9.4% for the T_4 RIA and from 9.3 to 13.5% for the T_3 RIA. Addition of significant quantities (100-400 ng %) of T_3 or T_4

Figure 4. Percentage of T_3* (o) or T_4* (⊛) bound to antibody on the Sephadex columns (pH 8.6) in relation to incubation times. The lower two curves show percentage of T_3* bound to antibody at two different pHs (pH 8.0, x; pH 7.4, ⊙).

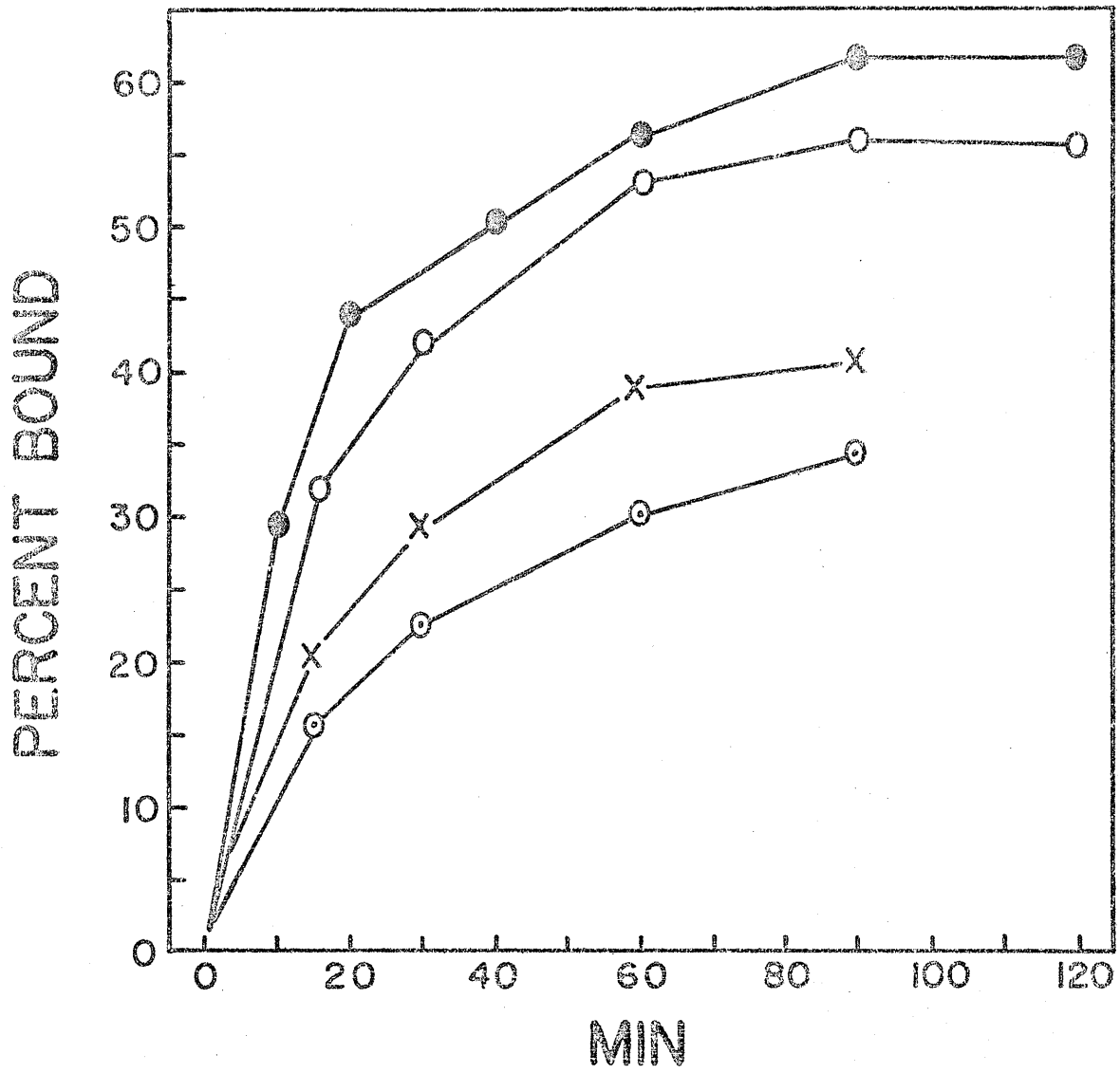

PERCENT BOUND
0
10
20
30
40
50
60
0
20
40
60
80
100
120
MIN

Figure 5. Percentage of radioactivity added to column as T_3* which was eluted with successive 1-ml eluates following incubation with T_3 antibody for 90 min. After 2 ml, the percentage eluted falls to values attributable to non-specific elution (antibody diluted to achieve 50-60% binding at '0' standard). T_4 RIA showed a similar pattern except non-specific elution was not evident (values represent the mean of duplicates).

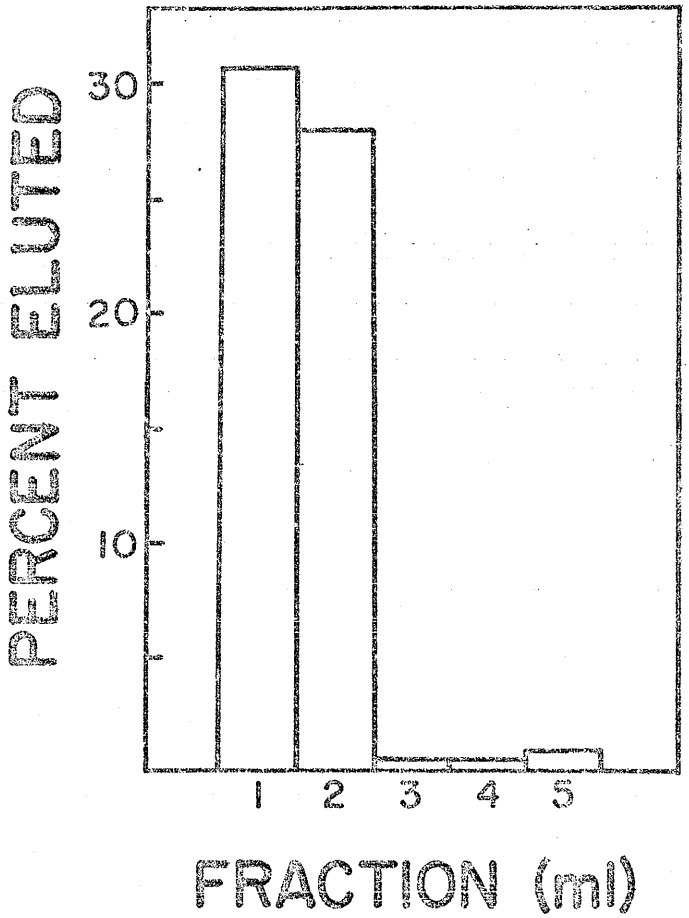

PERCENT ELUTED
30
20
10
1
2
3
4
5
FRACTION (ml)

Figure 6. Typical standard curves for T_4(●) and T_3(o).

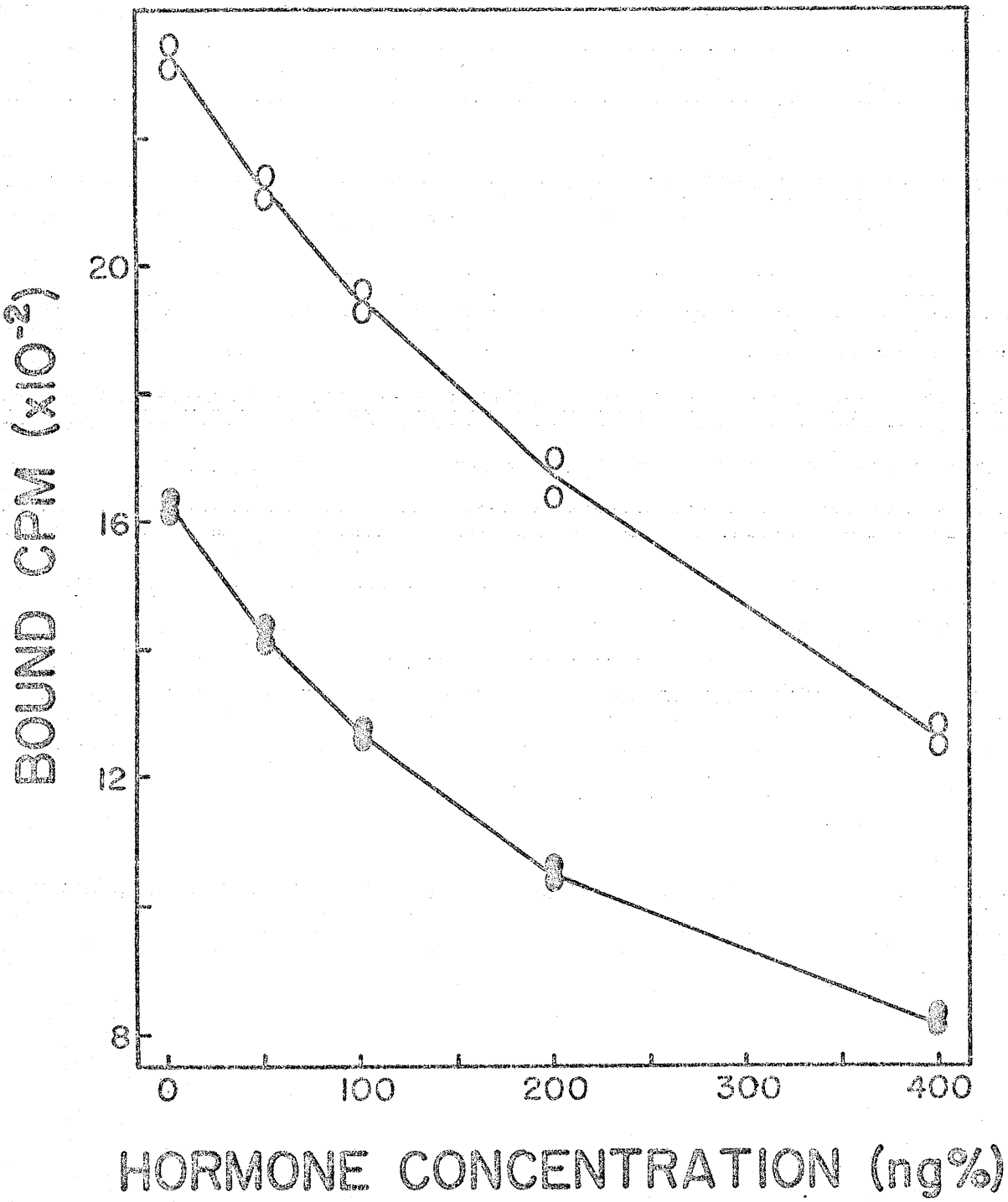

BOUND CPM (x10²)
20
16
12
8
0
100
200
300
400
HORMONE CONCENTRATION (ng%)

Figure 7. Standard curves for T_4 (●) and T_3 (o) plotted as F/B versus concentration.

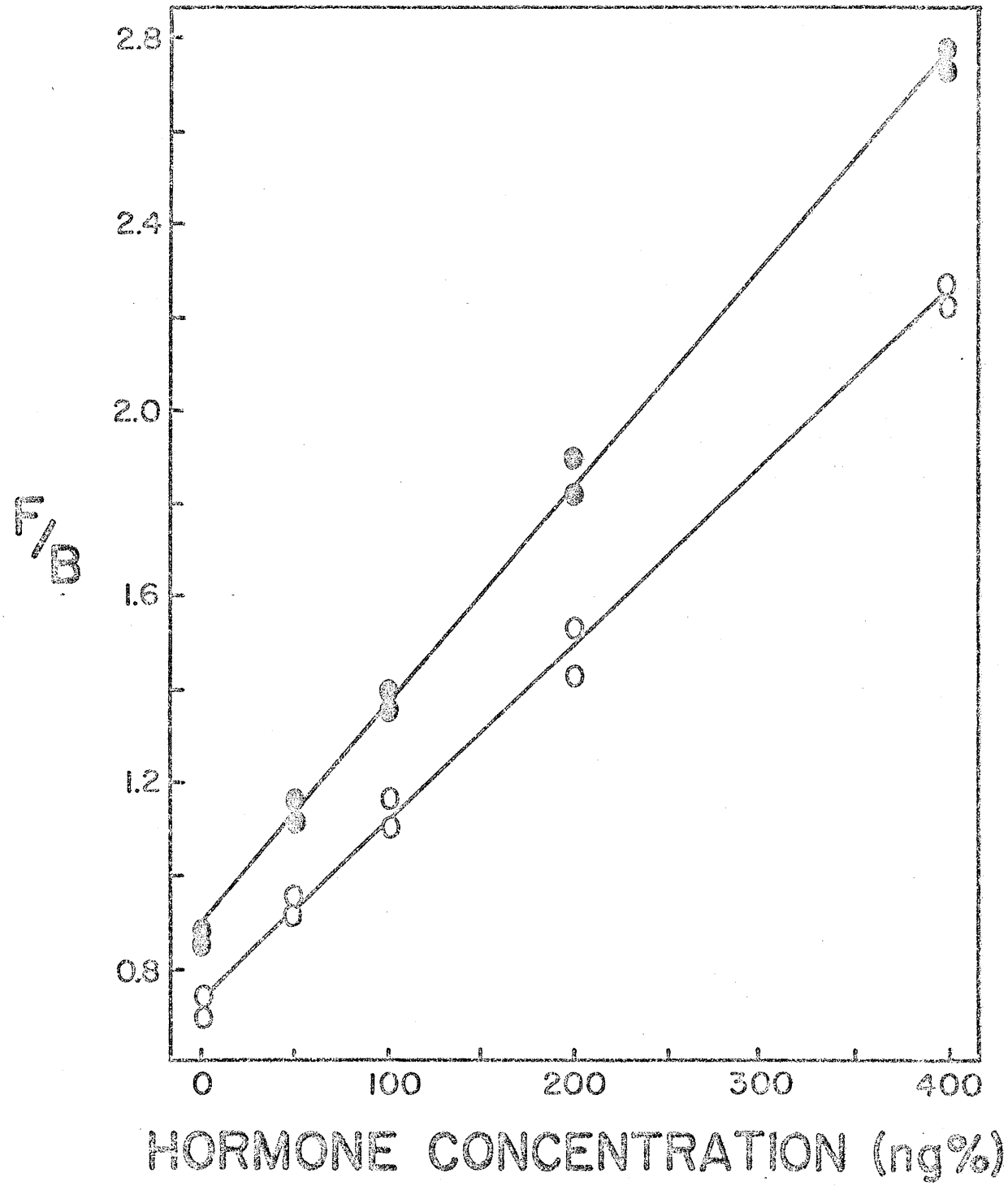

2.8
2.4
2.0
1.6
1.2
0.8
F/B
0
100
200
300
400
HORMONE CONCENTRATION (ng%)

Table 2. Mean values and ranges for the main variables derived from the regressions of 16 standard curves for T_4 and T_3 RIA. The standard curves were obtained by plotting F/B (the ratio of cpm in the free (Sephadex-bound) fraction to cpm in the antibody-bound fraction) versus hormone concentration (0-400 ng%) on the 'X' axis.

	T_4 RIA		T_3 RIA	
Parameter	Mean	Range	Mean	Range
Slope	0.0041	0.0035-0.0044	0.0037	0.0034-0.0042
Correlation Coefficient	0.990	0.980-0.993	0.994	0.983-0.999
Index of Precision	0.056	0.043-0.090	0.042	0.021-0.085
Detection Limit (ng%)	12.5	8.8 -22.0	9.5	4.9 -16.3

Table 3. Tests of "within-assay" reproducibility of T_4 and T_3 RIA on 0.1-ml samples from several rainbow trout plasma pools.

Assay	Plasma Pool	No. of Determinations	Hormone Concentration $\bar{X}$ (ng%)	SD	CV(%)
T_4	A	18	157	14.8	9.4
T_4	B	8	171	12.0	7.0*
T_4	C	8	552	28.1	5.1
T_3	D	8	130	13.5	9.6*
T_3	E	20	204	19.0	9.3
T_3	F	10	253	34.1	13.5

* Plasma pools B and D had 100 - 400 ng% of T_3 or T_4 respectively added to them prior to analysis.

to plasma pools B and D, respectively, did not interfere with RIA for either T_4 or T_3.

Table 4 shows the "between-assay' reproducibility obtained by making six to 11 duplicate determinations, on different days, on samples from trout or whitefish plasma pools which contained different endogenous levels of T_3 or T_4. The coefficient of variation for the RIA T_4 RIA ranged from 4.9 to 16.9% and for the T_3 RIA ranged from 5.1 to 15.9%.

Several human serum samples with varying T_3 levels were made available from the Winnipeg General Hospital, where a different form of radioimmunoassay was employed. There was a high correlation (0.96) between values obtained by the two assays (Fig. 8).

Table 5 shows the efficiency of T_4 and T_3 extraction from the plasma of four species of teleosts as determined by the method of Bauer et al. (1970). Mean extraction efficiencies for T_3 from unhemolyzed plasma samples were approximately 100%. This would give an overall extraction efficiency of 98% since 2% of the T_3 was eluted with the serum proteins or non-specifically with the first buffer wash (Fig. 3C). Mean extraction efficiency for T_4 was 99%. Hemolyzed samples had extraction efficiencies of 97.6% for T_3 and 97.9% for T_4.

Analytical recovery of T_4 or T_3 added to fish plasma and recovery of T_4 or T_3 in relation to sample volume are shown in Table 6. T_3 recoveries ranged from 88.5 to 116.0%; T_4 recoveries ranged from 98.9 to 120.0%.

To determine whether or not storage had any effect on rainbow trout plasma, duplicate iodothyronine analyses were performed on fresh plasma samples from six fish and subsequent duplicate analyses were

Table 4. Tests of "between-assay" reproducibility of T_4 and T_3 RIA conducted on 0.1 ml samples from rainbow trout or whitefish plasma pools.

Assay	Plasma Pool	No. of Assays	Hormone Concentration		
			$\bar{X}$ (ng%)	SD	CV%
T_4	Whitefish	7	115	19.4	16.9
T_4	Trout G	6	207	29.9	14.5
T_4	Trout H	6	612	29.8	4.9
T_3	Trout I	11	54	8.6	15.9
T_3	Trout H	6	328	16.7	5.1

Figure 8. Comparison of serum T_3 concentration estimated by Winnipeg General Hospital (WGH-RIA) and T_3 RIA using Sephadex columns. Correlation Coefficient = 0.96. Regression equation: RIA-WGH = 0.94 (T_3RIA) - 3.0.

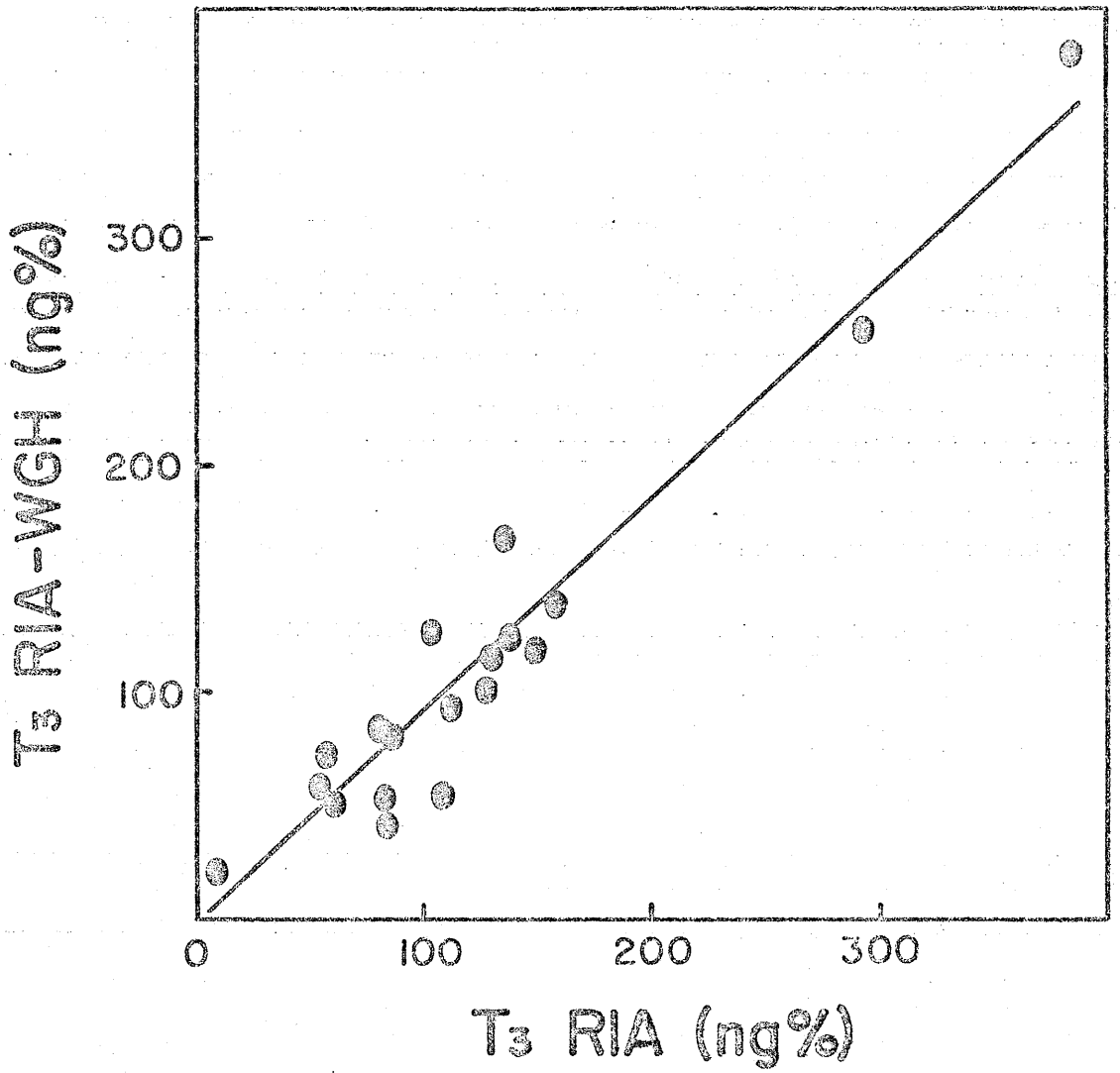

T3 RIA-WGH (ng%)
300
200
100
0
100
200
300
T3 RIA (ng%)

Table 5. Efficiency with which G-25 Sephadex columns extract T_3* and T_4* from plasma pools of four teleosts. Each value is a mean of 2-10 (n) determinations. A volume of 0.1-0.3 ml of a T_3* or T_4* plasma mixture was added to each column. The columns were then eluted with 3 ml (T_3RIA) or 4 ml (T_4RIA) of barbital buffer. Extraction efficiency = % of added T_3* or T_4 retained on columns after elution.

Species	n	Mean Extraction Efficiency (%)	
		T_3	T_4
Salmo gairdneri (Rainbow trout)	10	99.8	99.1
Ictalurus punctatus (Channel catfish)	2	100.3	99.1
Coregonus clupeaformis (Whitefish)	2	100.7	99.5
Catostomus commersoni (White sucker)	2	101.2	98.2
Hemolyzed rainbow trout plasma	8	97.6	97.9

Table 6. Percent recovery (%R) of T_3 and T_4 added to various heparinized rainbow trout plasma pools incubated for 10 min at 20°C with shaking; plasma volume varied from 0.05 - 0.30 ml. %R = (total hormone recovered - endogenous hormone) X 100/added hormone. Each determination is the mean of duplicates. Vs = volume (ml) of plasma sample added to the column. Values for each plasma pool were determined from single assays.

Assay	Plasma Pool	Endogenous Hormone (ng%)	Added Hormone (ng%)	Vs (ml)	%R
T_3	1	280	100	0.10	116.0
T_3	1	280	200	0.10	115.0
T_3	1	280	400	0.10	104.5
T_3	2	625	200	0.10	97.5
T_3	3	146	-	0.05	93.2
T_3	3	146	-	0.20	100.0
T_3	3	146	-	0.30	88.5
T_4	4	95	100	0.10	100.0
T_4	4	95	200	0.10	105.5
T_4	4	95	400	0.10	103.8
T_4	5	210	-	0.05	120.0
T_4	5	210	-	0.20	99.0
T_4	5	210	-	0.30	98.9

performed after one and 10 days of storage at -20°C. Two-way ANOVA showed no difference between T_4 levels in fresh or stored plasma (T_4 F = 3.48, 2 and 18 df; T_3F = 1.66, 2 and 18 df; Appendix Tables 1 and 2). As comparison, quadruplicate iodothyronine analyses performed on a human serum pool after one day of storage at -20°C did not differ from analyses of T_4 levels in fresh serum (t = 0.79; 6 df). However, freezing and storage of the same human serum pool for one day caused 12% increase in T_3 levels ($\overline{X}$ for fresh sera 152 ng %; $\overline{X}$ for frozen sera 170 ng %; t = 5.11, 6 df).

3. Diurnal variation in plasma T_4 and T_3.

Figure 9 shows the variation in plasma T_4, T_3, and T_4/T_3 at different times over two days. One-way ANOVA (Appendix Table 4) showed no significant F values for plasma T_4 (F = 1.04; 9 and 99 df), plasma T_3 F = 1.72; 9 and 97 df) or T_4/T_3 (F = 1.28; 9 and 96 df). Throughout the experiment the fish gained about one gram (final mean weight 25.9 g, SEM 0.6 g). The mean weights of the individual groups were similar (F = 0.91; 9 and 99 df).

Since no significant daily variation was found, the data were combined and possible influences of body weight and sex on hormone concentration investigated. Figures 10 and 11 show a correlation between ln plasma T_4 or T_3 and body weight of the fish. The correlation coefficients (T_4 = 0.301; T_3 = 0.455) were significant ($P < 0.05$). Regression analyses and F tests are given in Table 7.

The data were further analysed to compare hormone levels between male and female fish. The regression equations and F values for male and female fish are also given in Table 7. The covariance analyses showed

Figure 9. Plasma T_4, T_3 and T_4/T_3 in rainbow trout held on a 12-hr photoperiod. Vertical bars indicate the 95% confidence interval. Light and dark periods are indicated. Individual values for each group for plasma T_4, plasma T_3, T_4/T_3 and weight are given in Appendix Table 3.

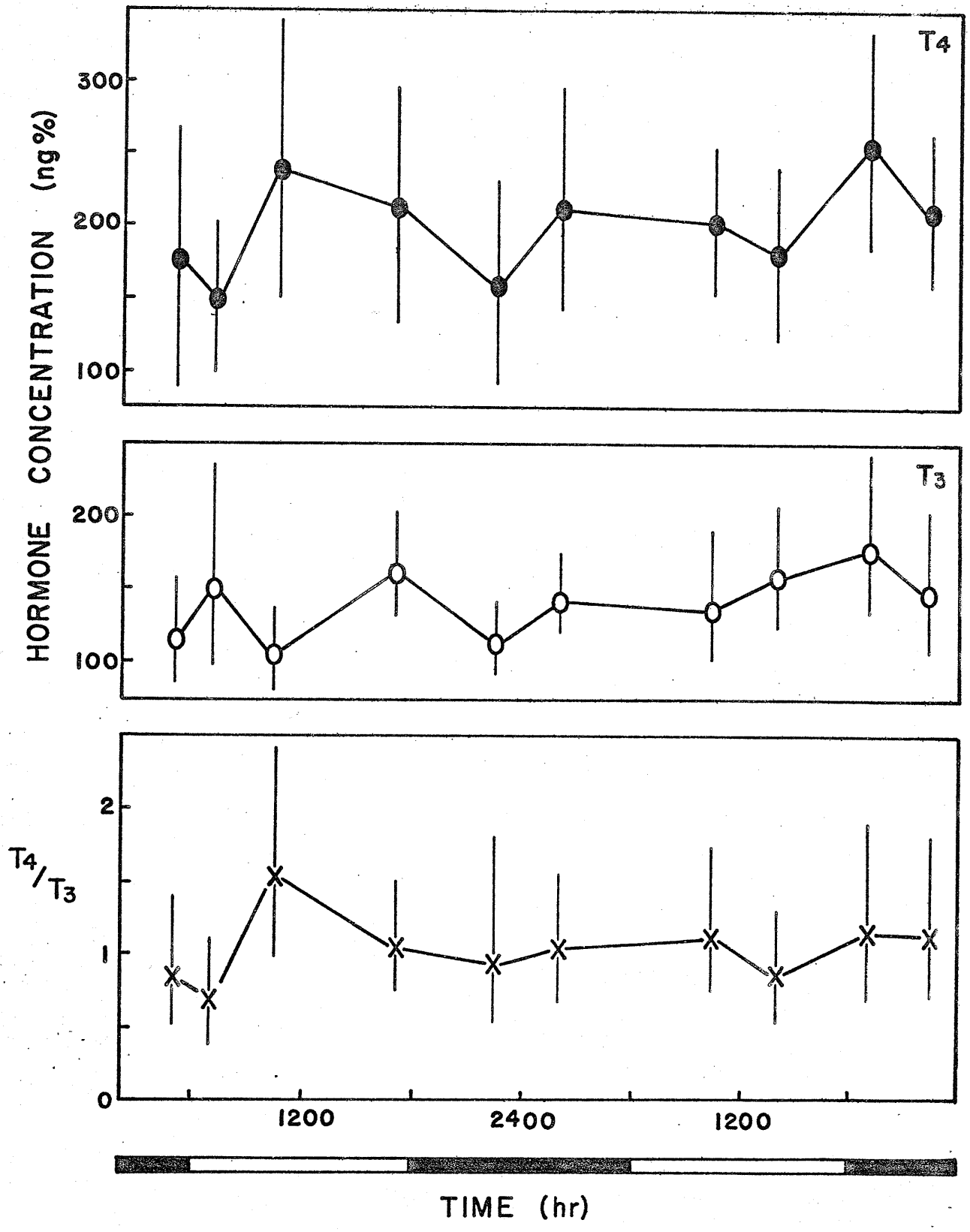

T4
300
200
100
HORMONE CONCENTRATION (ng%)
T3
200
100
T4/T3
2
1
0
1200
2400
1200
TIME (hr)

Figure 10. Correlation between plasma T_4 and body weight for male (x) and female (⊛) rainbow trout. Correlation coefficient = 0.301. Regression equation: LN HORMONE CONC = 0.029 (WEIGHT) - 4.372.

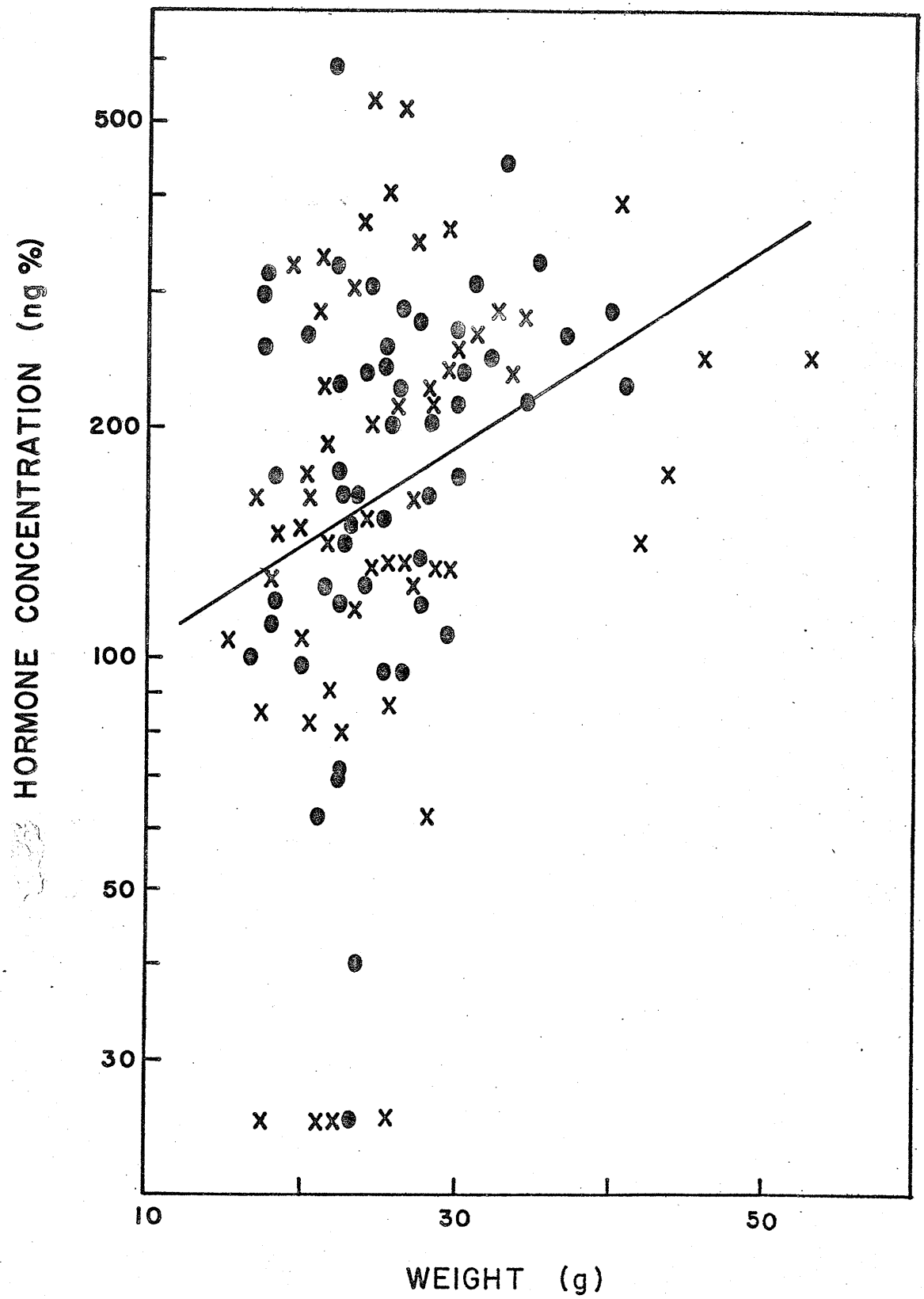

HORMONE CONCENTRATION (ng %)
500
200
100
50
30
10
30
50
WEIGHT (g)

Figure 11. Correlation between plasma T_3 and body weight for male (x) and female (●) rainbow trout. Correlation coefficient = 0.455. Regression equation: LN HORMONE CONC = 0.031 (WEIGHT) - 4.137.

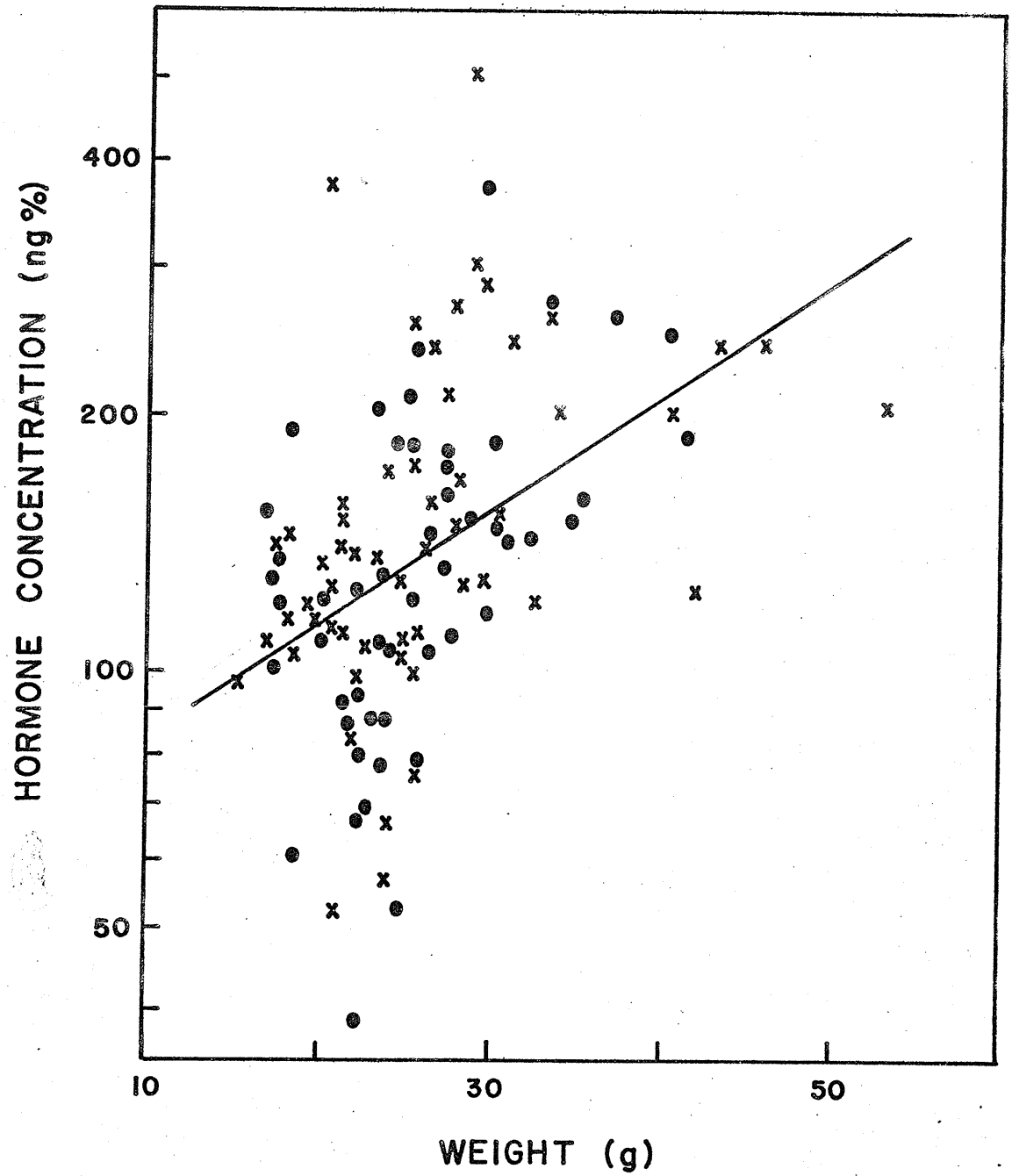

HORMONE CONCENTRATION (ng %)
400
200
100
50
10
30
50
WEIGHT (g)

Table 7. Correlation coefficients, regression values (slope b, intercept a), and F values from analyses of ln plasma T_4 and T_3 versus body weight. Appendix Tables 5 and 6 show the complete analyses.

Group	Regression a	Regression b	Correlation Coefficient	F value	Significance
T_4					
♂	4.339	0.0293	0.309	5.70	*
♀	4.388	0.0307	0.295	5.26	*
♂ + ♀	4.372	0.0297	0.301	10.68	* *
	Comparison of slopes ♂ vs ♀			0.06	NS
	Comparison of elevations ♂ vs ♀			0.43	NS
T_3					
♂	4.334	0.0246	0.421	11.05	* *
♀	3.854	0.0394	0.505	17.13	* *
♂+♀	4.137	0.0305	0.455	27.39	* *
	Comparison of slopes ♂ vs ♀			1.47	NS
	Comparison of elevations ♂ vs ♀			1.53	NS

* Level of significance 0.05

* * Level of significance 0.01

no difference between sexes for plasma T_4 and T_3. Furthermore, no difference between the mean weight of male (mean weight 26.3 g, SEM 1.0 g) or female (mean weight 25.5 g, SEM 1.0 g) trout was detectable (t= 0.635, 107 df).

The slopes of the combined regressions for both male and female fish versus plasma T_4 or plasma T_3 appeared similar (Table 7). However, the T_4 slope could not be compared with the T_3 slope because the variances were different between the regressions (F = 2.37 107 and 105 df; Appendix Table 7). These combined slopes indicate that plasma T_4 or T_3 increased by a factor of $e^{.03}$ per gram body weight.

There was no significant relationship between T_4/T_3 and body weight for male fish (r - 0.041; b -0.0052), female fish (r = -0.136; b = -0.0187), or male and female fish combined (r = -0.075; b = -0.0098).

4. Anaesthesia - immediate effects on plasma T_4 and T_3.

Both plasma T_4 and plasma T_3 were slightly but not significantly lower in fish subjected to MS 222 anaesthesia prior to bleeding (Table 8). There were no differences for T_4/T_3 or body weight between anaesthetized and non-anaesthetized fish.

5. Effect of anaesthesia and stress of cardiac injection on plasma T_4 and T_3.

Table 9 summarizes plasma T_4, plasma T_3 and T_4/T_3 values for control fish and fish injected with saline or sham-injected at two, six , and 12 hr pi. Two-way ANOVA (Appendix Table 8) showed no difference between sham-injected and saline injected fish in plasma T_4 (F = 0.07 , 1 and 58 df), plasma T_3 (F = 0.50 1 and 54 df) or T_4/T_3 (F = 0.18, 1 and 52 df). However, two-way ANOVA showed significant differences

Table 8. Mean values ($\overline{X}$) and 95% confidence intervals or standard error of the mean (SEM) for weight, plasma T_3, plasma T_4 and T_4/T_3 for 11 anaesthetized and 12 non-anaesthetized rainbow trout. Test statistics are given.

Group	Weight		T_4		T_3		T_4/T_3	
	$\overline{X}$	SEM	$\overline{X}$ (ng%)	95%	$\overline{X}$ (ng%)	95%	$\overline{X}$	95%
Anaesthetized	35.8	3.1	172	124-220	74	43-105	2.66	1.57-3.75
Non-anaesthetized	34.0	2.5	193	123-263	118	78-158	1.75	0.84-2.66
F-value	1.37		2.67		2.12		1.21	
Significance (P <0.05)	NS		NS		NS		NS	
t-value	0.45		0.52		1.81		1.74	
Significance (P <0.05)	NS		NS		NS		NS	

Table 9. Mean values ($\bar{X}$) and 95% confidence intervals or standard error of the mean (SEM) for weight, plasma T_4, plasma T_3 and T_4/T_3 for trout that had been injected with saline (I) or sham injected (S). Sampling occurred at 2, 6 and 12 hr pi. Both control groups were sampled 2 hr after the beginning of the experiment.

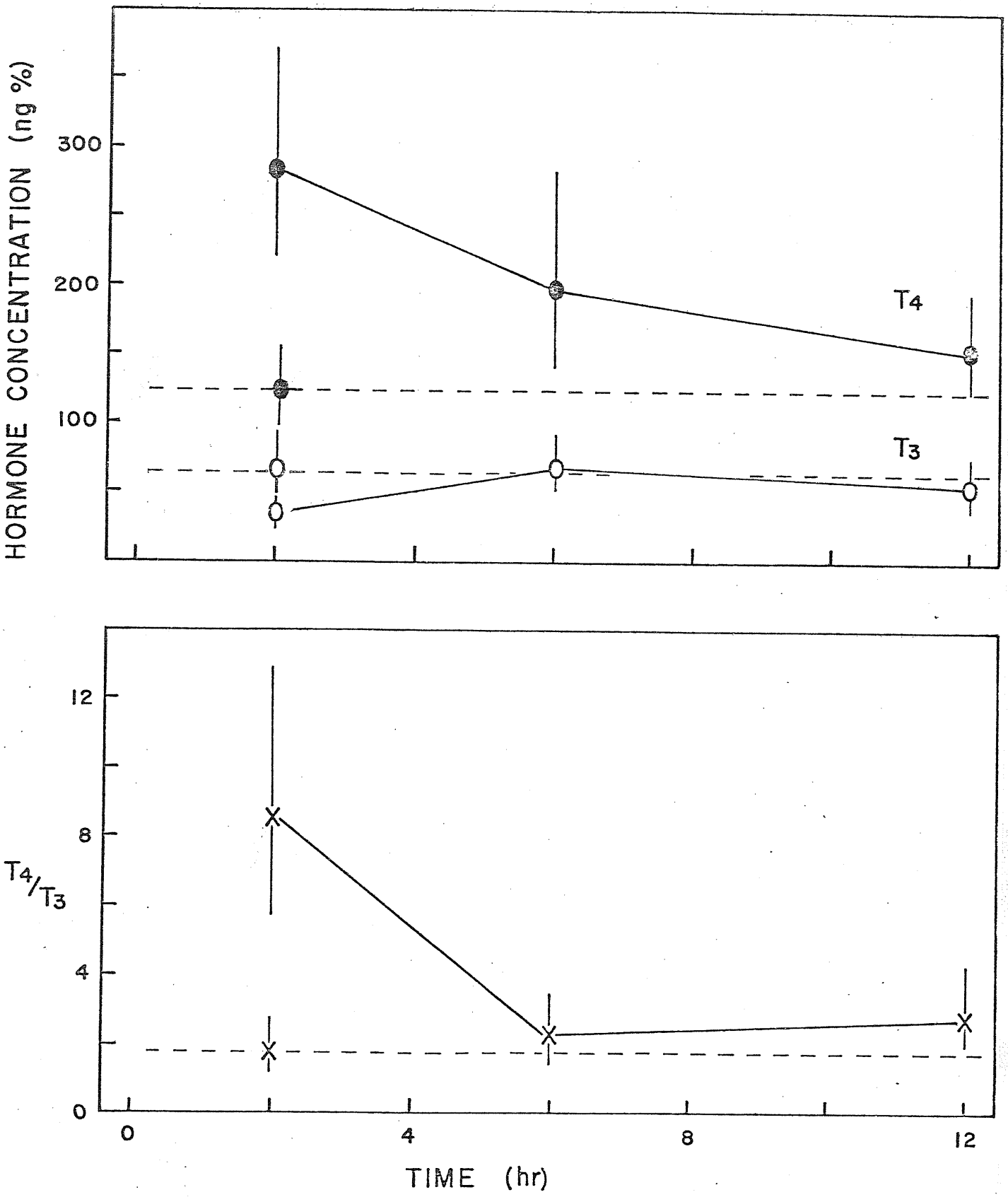
	Weight		T_4			T_3			T_4/T_3		
	$\bar{X}$ (g)	SEM	n	$\bar{X}$(ng%)	95%	n	$\bar{X}$(ng%)	95%	n	$\bar{X}$(ng%)	95%
Unhandled control	28.6	1.5	10	134	99-169	10	69	40-98	10	2.18	1.12-3.24
Handled control	27.5	1.3	10	141	91-191	10	67	25-105	10	2.91	1.42-4.40
2 hr-I	28.0	1.1	11	324	250-419	10	36	18-53	10	9.08	5.48-15.03
2 hr-S	25.4	1.1	11	253	155-411	10	36	15-57	10	8.24	4.03-16.87
6 hr-I	27.0	1.7	11	198	107-366	11	74	45-103	10	2.51	1.26-4.99
6 hr-S	26.7	1.1	11	200	128-313	11	66	34-97	10	2.18	1.70-2.81
12 hr-I	27.4	2.0	10	126	86-186	9	62	43-81	9	3.01	1.69-5.35
12 hr-S	27.2	0.9	10	188	144-246	9	50	16-84	9	2.90	1.70-5.09

between means at various times pi in plasma T_4 ($F = 5.03$, 2 and 58 df), plasma T_3 ($F = 4.34$, 2 and 54 df) and T_4/T_3 ($F = 13.83$, 2 and 52 df). Lack of interaction for plasma T_4 ($F = 1.35$, 2 and 58 df), plasma T_3 ($F = 0.11$, 2 and 54 df), and T_4/T_3 ($F = 0.02$, 2 and 52 df) indicates that the differences at various times pi are similar for both the saline-and sham-injected groups. No difference was evident in plasma T_4 ($t = 0.25$, df = 18), plasma T_3 ($T = 0.08$, df = 18) or T_4/T_3 ($t = 1.01$, df = 18) between the untouched control fish and the handled and fin-clipped controls.

Since no difference due to treatments (sham-versus saline-injection) or between handled and untouched controls was found, the two groups of fish (saline-and sham-injected) at each time were combined. These data are given in Figure 12. The combined data from the control groups were assumed to represent hormone concentrations at zero time and were assumed not to vary throughout the 12-hr experimental period. These assumptions are valid since the fish were held under identical conditions to fish in the diurnal study in which no daily fluctuations could be demonstrated.

Figure 12 shows that following heart injection of trout the plasma T_4 rose to a maximum by 2 hr pi and recovered to near control values by 12 hr pi. Plasma T_3 decreased slightly but significantly at 2 hr pi and recovered to control levels by 6 hr pi. T_3 fell slightly again at 12 hr pi. T_4/T_3 showed a marked increase at 2 hr pi returning to control values by 6 hr pi. One-way ANOVA followed by Newman-Keuls test (Appendix Table 10, 11) showed the following patterns:

Figure 12. Plasma T_4 (●), T_3(o), and T_4/T_3(X) from rainbow trout sampled at various times after cardiac injection. Control fish were sampled at 2 hr after beginning the experiment and the values (-----) were assumed not to vary. Vertical bars indicate 95% confidence intervals.


HORMONE CONCENTRATION (ng %)
300
200
100
T4
T3
T4/T3
12
8
4
0
0
4
8
12
TIME (hr)

Plasma T_4	Control	12 hr	6 hr	2 hr
Plasma T_3	2 hr	12 hr	Control	6 hr
T_4/T_3	Control	6 hr	12 hr	2 hr

(lines join groups whose means are statistically similar)

Mean weights of the four combined groups were similar (F = 0.50, 3 and 80 df).

6. Effect of seven days' starvation on plasma T_4 and T_3.

Plasma T_4, plasma T_3 and T_4/T_3 are given with statistics in Table 10. Plasma T_4 and T_4/T_3 decreased significantly in starved fish. No difference could be demonstrated for plasma T_3 between starved and fed fish. Although the initial weights of the two groups were similar, after seven days of food deprivation the mean weight of the starved group was 6.8 g less than those for fed fish.

7. Effect of time of starvation or feeding on plasma T_4 and T_3.

In contrast to the previous experiment, plasma T_4 (Fig. 13) did not change significantly due to feeding or starvation (F = 3.89, 1 and 40 df). However, T_4 was slightly lower in starved fish after three, seven and 14 days.

Also in contrast to Experiment 6, plasma T_3 (Fig. 13) differed between fed and starved fish (F = 5.12, 1 and 38 df). Two-way ANOVA also showed a significant interaction between feeding and starving and time (F = 8.58, 2 and 38 df). Newman-Keuls test, comparing the means from the fed (F) and starved (S) groups, gave the following pattern:

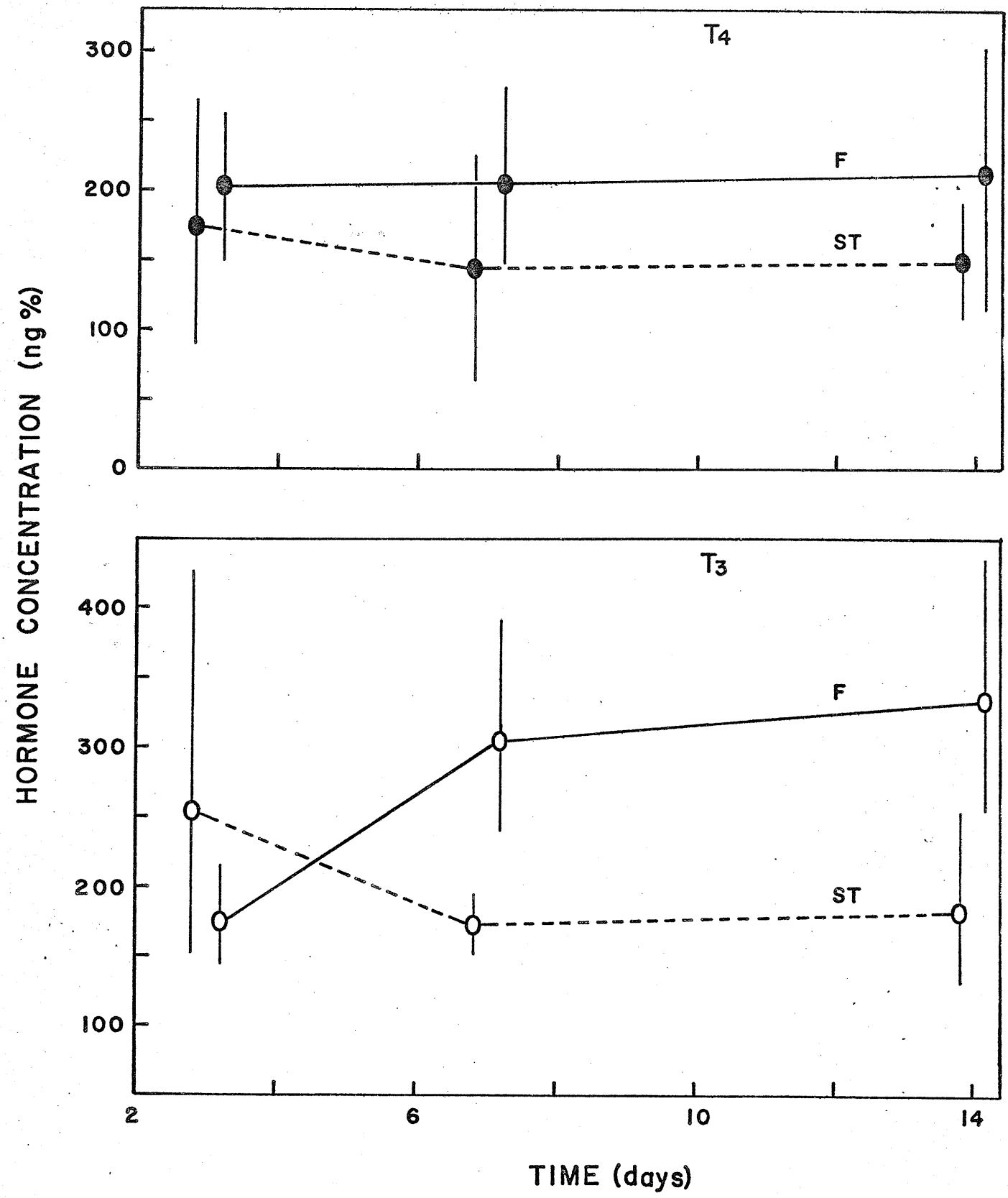
Table 10. Mean ($\overline{X}$) plasma T_4, T_3, T_4/T_3 and mean fish weights with 95% confidence intervals or standard error of the mean (SEM) for 11 fed and 13 starved rainbow trout.

Group	Weight		T_4		T_3		T_4/T_3[1]	
	$\overline{X}$ (g)	SEM	$\overline{X}$ (ng%)	95%	$\overline{X}$ (ng%)	95%	$\overline{X}$	95%
Fed	41.6	1.4	123	93-153	110	61-159	1.05	0.65-1.71
Starved	34.8	0.8	74	50-98	120	89-151	0.50	0.38-0.67
F-value	2.74		1.21		2.05		2.28	
Significance	NS		NS		NS		NS	
t-value	4.09		2.50		0.38		2.90	
Significance	*		*		NS		*	

* Level of significance 0.05

[1] Data transformed by $\log_{10}$ to reduce heterogeneity of variance in untransformed data.

Figure 13. Plasma T_4 (●) and T_3 (o) from fed (——) and starved (---) rainbow trout at various times after initiation of starvation. Vertical bars represent 95% confidence intervals. (Analyses and data summary are given in Appendix Tables 12, 13 and 14)


T4
300
200
100
0
F
ST
T3
400
300
200
100
F
ST
2
6
10
14
HORMONE CONCENTRATION (ng %)
TIME (days)

7 day-S 3 day-F 14 day-S 3 day-S 7 day-F 14 day-F

(lines join groups with statistically similar means)

In summary, the results show that plasma T_3 of fed fish increased significantly from three to seven days and remained elevated at 14 days. There was no difference in mean plasma T_3 levels for fish starved three, seven, or 14 days.

The 95 percent confidence interval for the three-day starved group (Fig. 13) was larger than for the other groups. Two hormone values recorded from the group starved three days were greater than 500 ng %. The mean excluding the two excessive values was 197 ng % (95% confidence = 149 - 260 ng%). The cause of the high values was unclear as the fish looked healthy and were the same size as the others in the group. Reanalysis by T_3 RIA confirmed their validity. There was considerable mortality during this experiment (12 died, 2 jumped out) and some irregularities in the water supply.

Two-way ANOVA (Appendix Table 12) showed no differences between mean T_4/T_3 or weights for each group.

8. Analysis of food for T_4 and T_3.

Mean extraction efficiency of added T_4* or T_3*was 97.5% (SEM 2.6%) and 101.5% (SEM 2.9%), respectively, for duplicate trials. EWOS trout grower contained 0.87 ng/g (SEM 0.12 ng/g) T_4 and 0.49 ng/g (SEM 0.16 ng/g) T_3. Addition of stable T_4 or T_3 to a second batch of food slurry after homogenization (10 ng/g) resulted in mean recoveries[a]

[a] $$\% \text{ Recovery} = \frac{(\text{Total hormone recovered} - \text{endogenous hormone})}{\text{added hormone}} \times 100$$

of 91.1% (SEM 2.9%) for T_4 and 110.8% (SEM 5.6%) for T_3 on duplicate trials.

DISCUSSION

1. RIA of plasma T_4 and T_3 in trout.

The T_4 and T_3 RIA procedures were developed as a modification of the Tetralute competitive binding assay for T_4, described by Higgs and Eales (1973). The principle of these RIAs is basically the same as that used in the Tetralute procedure, except that antisera to T_4 or T_3 replace thyroxine-binding globulin as the specific binding agent.

T_4 RIA will give more accurate measurements of teleost plasma T_4 than CPB assays. Alexander (1976) demonstrated that T_3 cross-reacts about 1/6 to 1/9 the extent that T_4 reacts with thyroxine-binding globulin. T_3 cross-reacts less than 1/100 the extent that T_4 does with the T_4 antisera. Under circumstances such as those encountered when maintaining trout on a high ration, T_4 levels are about 200 ng % whereas T_3 levels may be as high as 450 ng %. If 1/6 of the T_3 cross-reacts then T_4 levels could be over-estimated by as much as 75 ng % by the Tetralute method. In contrast to this previously used method, the T_4 and T_3 RIA procedures are highly specific. Addition of varying amounts of the 'other' iodothyronine did not interfere.

The use of Sephadex columns with antisera to T_3 has been previously employed in assays on human serum by Shimizu et al. (1973), Alexander and Jennings (1974b) and by Howorth and Marsden (1976) using the Ames T_3 kit. The use of Sephadex columns with antisera to T_4 has not been reported previously.

The values for "within" and "between" batch reproducibility are slightly higher for T_3 RIA than those previously reported using Sephadex

columns (Alexander and Jennings 1974b and Howorth and Marsden (1976). For the T_4 RIA the values are slightly higher than those reported for Sephadex (non-RIA) binding techniques (Braverman et al. 1971; Alexander and Jennings 1974a; Bastomsky et al. 1976) but compare well with RIA techniques using Sephadex for T_3.

The extraction efficiencies of T_4 and T_3 by Sephadex were similar to those previously reported in human and fish plasma (Higgs and Eales 1973; Alexander and Jennings 1974b). The extraction efficiency was not greatly influenced by hemolyzed samples.

The present methods using Sephadex offers other advantages. In many assay systems serum proteins that bind thyroid hormones compete with the T_4- or T_3- antisera giving erroneous results. This interference is usually reduced by adding to the assay system chemicals that inhibit T_4 or T_3 binding by the serum proteins. However, these inhibitors also interfere with antibody-binding (Burke and Eastman 1974). With Sephadex columns, the serum proteins are completely removed in the first buffer wash. In addition, the volume of plasma that can be added to each column is flexible and separation of the antibody-bound fraction from the free (Sephadex-bound) fraction is easy and nearly complete. Surks et al. (1972) reported no evidence for deiodination of T_4 to T_3 on miniature Sephadex columns. A disadvantage of Sephadex is that more antibody is required than for assays which incubate in an aqueous medium that is homogeneous. T_4 or T_3 is bound on the fixed Sephadex matrix and all the molecules are not accessible for antibody binding (Alexander and Jennings 1974b).

In this study, the use of T_4 antiserum and high-specific-activity

T_4* has permitted attainment of the low detection limit necessary for the study of plasma T_4 in teleosts. Values lower than the detection limits were infrequently encountered in healthy, unstressed fish. The method was convenient since with little modification it could be applied for T_3 measurement. Once Sephadex columns were set up the assays could be run quickly and economically. The quantities of T_4* and T_3* that were used were extremely small and the quantities of antibody used also small. Lyophilized antisera purchased in bulk reduced the cost further.

2. Influence of storage on plasma T_4 and T_3.

Packard et al. (1976), using the Tetralute method, noted that freezing and storage altered T_4 levels. Hagfish sera stored frozen for 5.5 days had lower T_4 levels than fresh sera. Watson and Lees (1973) reported that storage of human sera at -20°C for 3 months did not usually change T_4 levels by Tetralute, although T_4 in some sera decreased by 10-15%. The present results from freezing and storage of trout plasma support those of Watson and Lees. No change was detectable after 10 days storage for either T_4 or T_3 in trout plasma. Analysis of a human serum pool also showed no change for T_4. However a 12% increase for T_3 was detected in the same human serum pool. A high level of T_4 relative to T_3 exists in normal human sera (Ingbar and Woebar 1974). Consequently, although a small percentage of T_4 undergoing deiodination in the freezing and thawing process may be difficult to detect, the T_3 so generated may be sufficient to alter the small T_3 pool and create a significant increase in plasma T_3. In contrast to humans, the plasma T_4 levels in trout are generally similar to the T_3

levels. Thus, a large percentage of the circulating T_4 would have to be artifactually deiodinated to alter significantly T_3 levels.

3. Range of plasma T_4 and T_3 in immature trout.

Plasma T_4 and T_3 ranged from assay detection limits (12.5 ng % for T_4; 9.5 ng % for T_3) to 600 ng %. Most T_4 values fell between 150 and 250 ng % while most T_3 values fell between 50 and 200 ng %. These ranges are lower than estimates of plasma T_4 and T_3 based on PBI determinations in rainbow trout (Jacoby and Hickman 1966). In agreement with my data, Higgs and Eales (1973) found a range of 150 to 650 ng % for plasma T_4 in seven large ($\bar{X}$ = 343g) rainbow trout that were starved. However, they reported a higher plasma T_4 range for immature brook trout trout (0-1530 ng %). Leloup and Hardy (1976) also using RIA reported a similar range (60-580 ng %) to my values for plasma T_3 in rainbow trout and the eel, but plasma T_4 values were higher (260-1500 ng %). Since no record of size, physiological state, time of capture or assay procedure was given the reason for the difference is uncertain.

4. Diurnal variation.

The results indicated no diurnal fluctuations in plasma T_4, T_3 or T_4/T_3 in trout that had been starved for three days. A pattern with approximately a 16-hour interval appears to exist in the T_4 levels. However, high variability occurred between individual fish and the significance of the 16-hour pattern could not be determined.

Some authors believe the daily variations of thyroid hormone observed in man reflect changes in fluid distribution associated with postural changes (DeCostre et al. 1971; Vernikos-Danellis et al. 1972; Johns et al. 1975). Balsam et al. (1975) reported no overall changes

in total plasma protein in man but observed changes in thyroid binding capacities of plasma proteins. Since the changes were synchronous for T_3 and T_4 it was suggested that protein binding might be responsible. Fish, being continually supported by a buoyant medium, do not undergo major postural changes. Similarly, rats being quadrupeds, undergo less postural change during the day than man. Variations in plasma T_4 and T_3 are sometimes difficult to demonstrate in rats (Fukuda et al. 1975a). The large extrathyroidal pools of T_4 and T_3 that occur in most mammals may also be masking diurnal fluctuations in secretion rate or utilization (Fukuda et al. 1975a).

Starvation can "sham-hypophysectomize" animals (Aschkenasy et al) 1962). The fish in this study were starved for three days prior to sampling. Inability to detect diurnal changes in plasma thyroid hormones may be due to dampened TSH secretion. The effect of starvation cannot be ruled out without further study on pituitary function. Starvation for three days was felt necessary to eliminate effects due to feeding on the metabolism of thyroid hormones (Higgs 1974) or their biliary excretion (Eales and Sinclair 1974). Significant body wasting did not appear to be introduced.

5. Influence of sex.

No sex related variations could be detected for plasma thyroid hormones in immature trout. These results agree with Matty's (1960) findings that plasma PBI was not sex-dependent in the Bermuda parrot fish. Lewis and Dodd (1976) could demonstrate no differences in plasma T_4 or T_3 between male and female sharks.

Measurement of plasma hormone concentration gives no indication of hormone turnover or gland secretion. These may differ between sexes. However, Hoar and Eales (1963) found no difference between gland incorporation of radioactive iodide into plasma radioactive hormone for male and female goldfish. Also, the response of the thyroid gland to TSH stimulation did not differ between sexes in goldfish and brook trout (Ortman and Billig 1966; Chan and Eales 1976).

6. Influence of body weight.

Both plasma T_4 and T_3 were positively correlated with body weight. The relationship did not differ between sexes. Bray et al. (1976) reported a similar correlation for T_3 in man. Larger fish show proportionally greater thyroid hormone stores in the gland of the Bermuda parrot fish (Matty 1960; Matty and Thornburn 1970). Chan and Eales (1976) also found increased response to exogenous TSH treatment in larger brook trout. Eales (unpublished data) has confirmed that plasma T_4 and T_3 also increase with body weight in fed fish and over a greater size range.

7. Influence of anaesthesia.

No change occurred in plasma T_4 or T_3 in fish that were sampled immediately after anaesthesia or at two hours after anaesthesia. Thus light anaesthesia with MS 222 appeared to have little effect on plasma T_4 or T_3 in trout. These results are not supported by findings in man. Plasma PBI and T_4 increase following anaesthesia in man

(Oyama et al. 1969 a,b; Harland et al. 1974). Plasma T_3 in man decreases following anaesthesia (Brandt et al. 1976).

The reasons for these differences are unclear. Plasma T_4 or T_3 may not be as responsive to anaesthesia in fish as in man. Also the fish may not have been sedated (1-2 min) as heavily as humans undergoing operations. The chemical structure of MS 222 is more like local anaesthetics (Houston et al. 1976) than inhalation anaesthetics used for major surgery in mammals. Therefore, the type of anaesthetic may be responsible for the difference between fish and mammals. Finally, changes in circulating T_4 and T_3 could have been overlooked due to infrequent sampling.

8. Influence of surgical stress.

When trout were anaesthetized and stressed by cardiac puncture plasma T_4 doubled compared to control values at two hours pi. Plasma T_4 did not fully recover by 12 hours pi. Plasma T_3 decreased at two hours pi recovering to control values at six hours pi. In agreement with the data obtained in this study, serum T_3 falls with elective surgery in man (Bermundez et al. 1975; Burr et al. 1975a b; Brandt et al. 1976). However, serum T_4 levels in humans are generally unaltered or rise slightly (Bermundez et al. 1975; Brandt et al. 1976). Plasma T_4 of fish, following anaesthesia and surgical stress, appeared to follow the pattern outlined for anaesthesia in man (Harland et al. 1974). Perhaps the combination of anaesthesia and stress was required in fish. Further study using only surgically-stressed fish will help to determine whether or not the combination of anaesthesia and surgery was necessary to elevate plasma T_4.

9. Nutritional state.

i) Influence of dietary hormone. Little T_4 or T_3 was detected in EWOS trout grower (<1 ng/g). Recovery of added tracer was high and the recovery of added stable hormone was similar to the recovery of stable hormone recorded for radioimmunoassay. The variability encountered in recovery may be due to intraassay variability. However, it does appear that 10% of the added T_4 might be deiodinated to T_3 during the extraction procedure.

Feeding a three percent ratio to a 100-g trout would result in the ingestion of about 3 grams of food and about 2.6 ng T_4 or 1.5 ng T_3. Fed rainbow trout have degradation rates of 111 ng T_4 or 15.6 ng T_3 per 100 g fish daily (Eales, unpublished data). Assuming complete uptake of ingested hormone, 2.3% of daily T_4 or 9.6% T_3 requirement could enter via the food. Absorption of T_4 from the intestinal contents in vivo was found to be less than 15% for brook trout (Eales and Sinclair 1974). If the same extent of uptake existed for rainbow trout about 0.5% of the daily T_4 requirement would be contributed by the food. Digestion also takes place over the course of hours. Therefore it is unlikely, even if complete uptake occurred, that the T_4 or T_3 would alter plasma hormone levels significantly.

Strict quality control was not maintained and subsequent batches of food used after the analysis may have contained differing amounts of hormone.

ii) Influence of starving and feeding. Experiment 4 showed lower plasma T_4 in starved fish. In Experiment 5, T_4 showed a downward trend

due to starvation but was not significantly lower in starved fish. The inability to show significant reductions due to starvation in Experiment 5 is unclear, but could be related to smaller sample sizes of the groups and higher individual variability than observed in Experiment 4. Overall, the results agree with findings in mammalian studies, where PBI and T_4 either show no change or a slight decrease in starved animals (Schatz et al. 1967; Merimee and Fineberg 1976). Higgs (1974) also sometimes observed lower plasma T_4 in brook trout. Packard et al. (1976) and Osborn and Simpson (1972) reported lower plasma T_4 in hagfish and plaice after capture. They attribute some of the decreases due to starvation.

Starvation for seven days did not alter plasma T_3 levels in Experiment 4. In Experiment 5, starvation for three, seven, or 14 days did not significantly change plasma T_3 levels when compared to the fed control fish for three days. However, circulating T_3 was higher in the fed control fish for seven and 14 days. The reason for the higher levels between the fed control fish for seven and 14 days and the fed control for three days is unclear, but may be related to the length of time the fish were maintained on a higher ration. Prior to experimentation the fish were fed a near maintenance diet (1.0 - 1.5%). Then the fish were fed a three percent ration for ten days before half the fish were starved. If the plasma T_3 levels had not completely adjusted to the increased ration in the 10 days then the observed results might occur. Thus, it appears that at least 17 days (10 days conditioning + seven days experimentation) may be required to allow circulating T_3 to adjust to a major dietary shift. In Experiment 4

the fish were only fed a higher ration for four days prior to experimentation and no change in plasma T_3 levels occurred.

Supporting the results described here, Osborn and Simpson (1972) found lower T_3 in the plaice after starvation. Results from studies in mammals always show lower plasma T_3 after starvation (Portnay et al. 1974; Chopra et al. 1975a; Vagenakis et al. 1975).

T_4/T_3 was significantly higher in fed fish in Experiment 4 and slightly higher (NS) in the fed fish at day three in Experiment 5. Different T_4/T_3 at these times might indicate changing metabolism in fed fish. The similar T_4/T_3 in fed and starved groups at seven and 14 days may also mean the fish have adjusted to the higher ration. The results of Experiment 5 must be viewed with some reserve since unaccountable, high fish mortality and high variation in some of the plasma hormone levels were found. Repeating the experiment could be worthwhile.

A weight correlation with plasma T_4 and T_3 has been previously shown to exist in rainbow trout. Therefore a criticism can be made of the comparison of T_4 and T_3 between fed and starved fish. The differences in plasma thyroid hormones between fed and starved fish might be due to size differences since fed animals grow and starved animals lose weight. Note that fed fish have a non-physiological weight advantage, since the fed groups were sampled one hour after feeding. Little digestion would have occurred during this time and, as an example, a 50 g fish fed a three percent ration would contain 1.5 g extra weight as food. Residual food was observed in the intestine up to three days after initiation of starvation. If 0.5 g (mean value from five fish) of food from the previous day were left, a false increase of two grams could be recorded for the body weight of each animal. The largest

difference in mean weight between fed and starved fish existed in the 14 day groups from Experiment 5 (19.1g). The mean plasma T_3 differed by 151 ng % in these fish, and the weight related correlation can only account for 13% of the difference in circulating T_3.

10. Possible mechanisms altering plasma T_4 and T_3 in fish.

The mechanisms whereby plasma thyroid hormones are changed in fish have not been established. Some possible mechanisms are discussed below and include alterations in deiodination, clearance rates, blood volume, plasma binding of thyroid hormones, thyroid or pituitary gland activity, peripheral redistribution of hormone, and iodide availability.

Deiodination of T_4 to T_3 readily occurs in brook and rainbow trout (Higgs 1974; Eales unpublished data). The higher T_4 and lower T_3 following anaesthesia and surgical stress may be the result of decreased peripheral conversion. Deiodination is thought to activate the hormone in mammals (Pittman 1971) and perhaps active thyroid hormones are not needed in such large quantities in times of stress. Deiodination also decreases in starved fish (Higgs 1974). The results showed T_4 was unaltered or slightly reduced in starved fish. Thus something more than a simple reduction in peripheral conversion must be occurring in starved fish.

Feeding brook trout increased metabolic clearance for T_4 and T_3 and sometimes T_4 degradation rates (Higgs 1974). Increased clearance of plasma T_4 or T_3 during feeding would likely cause, all other factors being constant, lower hormone pools. Thus, increased metabolic clearance

cannot explain unaltered or elevated plasma hormone levels found in fed fish. Reduced T_4 clearance during stress might cause the high T_4 values observed in anaesthetized and injected fish. However, higher T_3 clearance must also occur to explain decreased plasma T_3 and this seems unlikely. If major changes in clearance rates are altering circulating T_4 or T_3 other factors must also be involved.

Anaesthesia with MS 222 or stress-related variables can cause hemoconcentration in fish (Stevens 1968; Houston et al. 1971; Fletcher 1975). An increase due to hemoconcentration in the plasma concentration of proteins which bind thyroid hormones might be responsible for increased T_4 levels. However this idea cannot explain unaltered or decreased T_3 levels. Hemodilution during starvation could cause lower plasma T_4 or T_3 levels. Hemodilution during starvation seems unlikely. Weinberg et al. (1976) found blood volume did not change during starvation of the kissing gourami. Love (1970) reported that blood volumes were unaltered or reduced in starved fish.

O'Connor et al. (1974) found high-frequency fluctuations of plasma T_4 in man. These short-duration changes (20 min) would be difficult to demonstrate in fish of the size used here, without larger numbers and more frequent sampling times. This type of change might be responsible for some of the variability observed between individual fish. Also Vernikos-Danellis et al. (1972) and Balsam et al. (1975) noted high individual variability for plasma thyroid hormones in man. Marked intraspecific variation exists in teleost plasma protein levels and in electrophoretic patterns of rainbow trout serum (Thurston 1967; Feeny and Brown 1974). Falkner and Eales (1973) showed that 99% of

circulating T_4 or T_3 was bound to plasma proteins. Alterations in the proteins that bind thyroid hormones could cause some of the observed variability.

The basis of the positive correlation of plasma T_4 and T_3 with weight is unclear, but it may be related to greater thyroid hormone binding by the plasma proteins of larger fish. Booke (1964) reported that higher plasma protein levels occurred in larger and older rainbow trout. If the plasma proteins that bind T_4 and T_3 also increased in quantity or binding capacity in larger and older fish then a positive correlation between plasma T_4 and T_3 and body weight would occur. The data support the preceding hypothesis. For example, the regression lines for plasma T_4 and T_3 versus body weight have almost identical slopes and T_4/T_3 showed no correlation to fish weight. Since the same plasma proteins likely bind T_4 and T_3 (Falkner and Eales 1973), any increase in their quantity or capacity would result in similar changes in both plasma T_4 and T_3. As well, no change in T_4/T_3 would be expected. Biologically, it is unclear why hormone levels increase with fish weight. Perhaps increased hormone reserves are required in larger fish for sexual maturation or other metabolic needs.

Furthermore, plasma protein binding of thyroid hormones is reduced during starvation in mammals and may be responsible for lower hormone levels in the starved state (Ingemble et al. 1974; Pain and Phillips 1976). Robertson et al. (1961) and Love (1970) reported reduced plasma protein levels in starved fish. If the proteins that bind T_4 and T_3 in the plasma were also lower in starved fish, reduced amounts of circulating T_4 and T_3 would be found. Changes in plasma binding of T_4 or T_3 seem unlikely in surgically-stressed fish. Plasma T_4 increased

while T_3 decreased. If the same plasma proteins bind T_4 and T_3 in fish, then preferential differences in the binding of one hormone compared to the other appear unlikely.

Another mechanism decreasing T_4 and T_3 in starved fish could involve reduced thyroid gland activity accompanied by pituitary reduction of TSH. Fontaine and Fontaine (1956) and Bonnet (1970) found that prolonged starvation lowered thyroid gland activity in rainbow trout and mullet. Gland activity is also reduced in starved mammals (Pipes et al. 1960; Grossie and Turner 1962). Reduced pituitary secretion of TSH during starvation has not been demonstrated in fish but has been shown in mammals (D'Angelo 1951; Shambaugh and Wilber 1974; Vinik 1975). Lower TSH production by the pituitary results in lower secretion of hormones by the thyroid.

Thyroid gland activity increases following moderate surgery in man (Harland et al. 1972; Kirby et al. 1973). Since the trout thryoid gland appears to secrete only T_4 (Chan and Eales 1975; Hunt personal communication) higher gland activity might explain higher T_4 levels in surgically-stressed trout.

Harland et al. (1974) showed that T_4 bound in the liver of man was released into the circulation following anaesthesia and surgery. Thus, altered hepatic distribution might account for the high T_4 observed in stressed fish. In support of this idea, Kumar et al. (1968) found that prednisone, a synthetic steroid similar to cortisone reduces hepatic volume and metabolism of T_4. Corticosteroids also reduce conversion of T_4 to T_3 (Chopra et al. 1975b; Burr et al. 1976). Surgery and anaesthesia cause elevated corticosteroids in teleosts (Fagerlund

1967; Wedemeyer 1969; Fryer 1975). Elevated natural steroids may be altering the peripheral T_4 distribution in surgically-stressed trout.

Food deprivation also causes iodide deprivation. Much of the iodide content in the trout plasma depends upon dietary iodide content (Gregory and Eales 1975). Brook trout blood iodide levels fall rapidly as a result of starvation. Critical levels of iodide required for normal hormone synthesis and metabolism have not been outlined in fish. Low levels of iodide cause reduced hormone levels in rodents (Fukuda et al. 1975b ; Chapman et al. 1976) and may also cause lower plasma T_4 and T_3 in teleosts.

Investigation of the preceding mechanisms will provide greater understanding of the factors or combination of factors contributing to alterations in plasma T_4 and T_3 in fish.

11. Physiological significance of changes in plasma T_3 or T_4.

In view of the many options available, carefully designed experiments are necessary to isolate factors contributing to alterations in plasma thyroid hormones. This study has shown that diurnal fluctuations do not occur in starved, immature trout held under laboratory conditions. It also shows that individual heterogeneity and stress or the inability to combat stress caused differences in plasma T_4 and T_3 in trout.

Lack of a daily fluctuation in laboratory fish allows sampling at any time during the day. For wild fish, which encounter more variable conditions, a diurnal variation may occur. Further study in this area might prove worthwhile.

Elimination of some of the individual heterogeneity may prove to

be a difficult problem. Sex appears to have little influence on plasma T_4 and T_3 in immature trout. By carefully choosing healthy fish of uniform size some of the variability in plasma thyroid hormones can be reduced. Genetic differences between fish (Montana stock versus Idaho stock) could have altered the results of the anaesthesia-injection stress experiment since a diurnal rhythm may exist in Montana fish. Experiments comparing different genetic stocks might prove interesting.

Starvation and physical injury may be regarded as two forms of stress. These two types of stress altered plasma T_4 or T_3 of trout in different ways. Physical injury caused elevation of plasma T_4, whereas starvation sometimes results in significant reductions in plasma T_4 and T_3. The mechanisms whereby these two types of stress change plasma T_4 and T_3 remain to be resolved for fish. Few generalizations can be made until many other forms of stress and their effects on teleosts thyroid function have also been investigated. Crowding, temperature, water quality, disease and various sampling procedures appear to be stress factors that might warrant further investigation. Thus it appears that careful consideration must be given to stress conditions that may affect thyroid function in teleosts before meaningful physiological data can be collected.

SUMMARY

1. Radioimmunoassay techniques can be successfully applied to measurement of circulating T_4 and T_3 in rainbow trout. The techniques are reproducible and capable of detecting physiological changes in plasma thyroid hormones.

2. Rainbow trout plasma samples can be stored at -20°C for at least 10 days prior to assay without significant degradation or loss of thyroid hormones.

3. Plasma T_4 in immature rainbow trout was 1/100 of the levels found in man. Plasma T_4 in trout ranged from assay detection limit (12.5 ng %) to 600 ng %. Plasma T_3 was similar to that in humans and other vertebrates but appeared more variable in rainbow trout. Depending on physiological and nutritional state of the fish, plasma T_3 ranged from assay detection limit (9.5 ng %) to 600 ng %.

4. Starved trout exhibited no diurnal variation in plasma T_3 or T_4.

5. High variability in plasma thyroid hormones existed among individual fish. The variability could not be entirely explained by the factors investigated here.

6. No difference in plasma T_4 or T_3 could be demonstrated between male and female trout.

7. A positive correlation between fish weight and plasma T_4 or T_3 was evident.

8. Light anaesthesia with MS 222 did not alter circulating thyroid hormones when given immediately before bleeding or two hours before bleeding.

9. Anaesthesia and injection doubled plasma T_4 while plasma T_3 was slightly but significantly lowered. These differences may be due to decreased peripheral conversion of T_4 to T_3 or to peripheral redistribution of T_4.

10. A test on a single batch of EWOS food revealed concentrations of 0.87 ng/g T_4 and 0.49 ng/g T_3. Ingestion of food containing these concentrations would not likely have a significant influence on plasma T_4 or T_3.

11. Starvation sometimes reduced plasma T_4 in trout.

12. Feeding a high ration for 17 days resulted in higher plasma T_3 in trout.

13. Stress factors can influence plasma T_4 or T_3 in rainbow trout and should be considered when designing experiments to measure thyroid function.

LITERATURE CITED

Alexander, N.M. 1976. Semi-automated competitive protein binding analysis of serum thyroxine on reusable Sephadex columns and its advantages over radioimmunoassay. Clin. Chim. Acta, 69: 293-298.

Alexander, N.M., and J.F. Jennings. 1974a. Analysis for total serum thyroxine by equilibrium competitive protein binding on small, reusable Sephadex columns. Clin. Chem. 20: 553-559.

Alexander, N.M., and J.F. Jennings. 1974b. Radioimmunoassay of serum triiodothyronine on small, reusable Sephadex columns. Clin. Chem. 20: 1353-1361.

Aschkenasy, A., B. Nataf, C. Piette, and M. Sfez. 1962. Le fonctionnement thyroiden chez de rat carence en proteines. Ann. d'Endocrinol. (Paris), 23: 311-332.

Auerbach, N. 1963. Diurnal variation in the level of serum protein-bound iodine in humans. Hartford Hosp. Bull. 18: 168-175.

Azukizawa, M., H.B. Roohk, J.J. DiStefano, and J.M. Hershman. 1975. Diurnal rhythm of plasma thyrotropin, triiodothyronine and thyroxine levels in rats. Clin. Res. 23: A92.

Balsam, A., C.R. Dobbs, and L.E. Leppo. 1975. Circadian variations in plasma concentrations of thyronine and triiodothyronine in man. J. App. Physiol. 33: 644-648.

Ban, Y. 1972. A study on the diurnal variation in the level of serum thyroid hormone. Endocrinol. Japon. 19: 594.

Barrington, E.J.W., N. Barron, and D.J. Piggins. 1961. The influence of thyroid powder and thyroxine upon the growth of rainbow trout (Salmo gairdneri). Gen. Comp. Endocrinol. 1: 170-178.

Bastomsky, C.H., J.M. Wyse, and P.V.N. Murthy. 1976. Assay of serum thyroxine by competitive protein binding: reuse of disposable Sephadex columns. Clin. Biochem. 9: 89-92.

Bauer, R., L.A. Schick, B.F. Phillips, and C.O. Rupe. 1970. Use of Sephadex G-25 in estimation of total serum thyroxine. Annu. Meet. Am. Assoc. Clin. Chem., Buffalo, N.Y.

Bermudez, F., M.I. Surks, and J.H. Oppenheimer. 1975. High incidence of decreased serum triiodothyronine concentration in patients with nonthyroidal disease. J. Clin. Endocrinol. Metab. 41: 27-40.

Bonnet, B. 1970. Thyroide et milieu intérieur chez Mugil sp. Influence de la nutrition et de l'environment. Ph.D. Thesis, Université d'Aix-Marseille, France.

Booke, H.E. 1964. A review of variations found in fish serum proteins. N.Y. Fish Game J. 11: 45-57.

Brandt, M., H. Kehlet, J.M. Hansen, and L. Stonsted. 1976. Serum triiodothyronine and surgery. Lancet, 1: 791.

Braverman, L.E., A.G. Vagenakis, A.E. Foster, and S.H. Ingbar. 1971. Evaluation of a simplified technique for the specific measurement of serum thyroxine concentration. J. Clin. Endocrinol. Metab. 32: 497-502.

Bray, G.A., D.A. Fisher, and I.J. Chopra. 1976. Relation of thyroid hormones to body-weight. Lancet, 1: 1206-1208.

Brown-Grant, K., G.W. Harris, and S. Reichlin. 1954. The effect of emotional and physical stress on thyroid activity in the rabbit. J. Physiol. 126: 29-40.

Brown-Grant, K., and J. Pethes. 1960. The response of the thyroid of guinea pig to stress. J. Physiol. 151: 40-50.

Burke, C.W., and C.J. Eastman. 1974. Thyroid hormones in Radioimmunoassay and saturation analysis edited by P.H. Sönksen. Br. Med. Bull. 30: 93-99.

Burr, W.A., E.G. Black, R.S. Griffiths, P. Prince, and D.B. Ramsden. 1975a. Thyroid response to acute illness. Clin. Sci. Mol. Med. 49: 22P.

Burr, W.A., R.S. Griffiths, E.G. Black, R. Hoffenberg, H. Meinhold, and K.W. Wenzel. 1975b. Serum triiodothyronine and reverse triiodothyronine concentrations after surgical operation. Lancet, 2: 1277-1279.

Burr, W.A., D.B. Ramsden, R.S. Griffiths, E.G. Black, and R. Hoffenberg. 1976. Effect of a single dose of dexamethasone on serum concentration of thyroid hormones. Lancet, 2: 58-61.

Chan, H.H., and J.G. Eales. 1975. Identification of iodoamino acids in the plasma of brook trout, Salvelinus fontinalis (Mitchill). Can. J. Zool. 53: 97-101.

Chan, H.H., and J.G. Eales. 1976. Influence of bovine TSH on plasma thyroxine levels and thyroid function in brook trout, Salvelinus fontinalis (Mitchill). Gen. Comp. Endocrinol. 28: 461-472.

Chapman, R.S., P.G. Malan, and R.P. Ekins. 1976. Influence of dietary iodine on thyroid hormone levels secreted from the mouse thyroid lobes in vitro. J. Endocrinol. 69: 6 -7P.

Chavin, W. 1956. Thyroid distribution and function in goldfish Carassius auratus L. J. Expt. Zool. 133: 259-280.

Chopra, I.J., U. Chopra, S.R. Smith, M. Reza, and D.H. Solomon. 1975a. Reciprocal changes in serum concentrations of 3,3',5'-triiodothyronine (T_3) in systemic illness. J. Clin. Endocrinol. Metab . 41: 1043-1049.

Chopra, I.J., and S.R. Smith. 1975. Circulating thyroid hormones and thyrotropin in adult patients with protein-calorie malnutrition. J. Clin. Endocrinol. Metab. 40: 221-227.

Chopra, I.J., D.E. William, J. Orgiazzi, and D.H. Solomon. 1975b. Opposite effects of dexamethasone on serum concentration of 3,3',5'-triiodothyronine (reverse T_3) and 3,3',5-triiodothyronine (T_3). J. Clin. Endocrinol. Metab . 41: 911-920.

Collicutt, J.M., and J.G. Eales. 1974. Excretion and enterohepatic cycling of ^{125}I-L-thyronine in channel catfish, Ictalurus punctatus Rafinesque. Gen. Comp. Endocrinol. 23: 390-402.

D'Angelo, S.A. 1951. The effect of acute starvation on thyrotrophic hormone level in the blood of rat and mouse. Endocrinology, 48: 341-343.

Deb, C., N. Mallick, and V. Goswani. 1973. Effect of different percentages of protein intake on thyroid activity, protein bound, total thyroxine, and free thyroxine level of blood of rats. Endokrinologie, 62: 321-326.

De Costre, P., U. Buhler, L.J. De Groot, and S. Refetoff. 1971. Diurnal rhythm in total serum thyroxine levels. Metabolism, 20: 782-791.

Ducommun, P., E. Sakiz, and R. Guillemin. 1966. Lability of plasma TSH levels in the rat in response to non-specific exteroceptive stimuli. Proc. Soc. Expt. Biol. Med. 121: 921-923.

Eales, J.G., and D.A.R. Sinclair. 1974. Enterohepatic cycling of thyroxine in starved and fed brook trout (Salvelinus fontinalis) (Mitchill). Comp. Biochem. Physiol. 49A: 661-672.

Ekins, R.P. 1974. Basic principles and theory. In Radioimmunoassay and Saturation Analysis. Edited by P.H. Sönksen. Br. Med. Bull. 30: 3-11.

Fagerlund, U.H.M. 1967. Plasma cortisol concentration in relation to stress in adult sockeye salmon during the freshwater stage of their life cycle. Gen. Comp. Endocrinol. 8: 197-207.

Falkner, N.W., and J.G. Eales. 1973. Investigation of iodothyronine binding to plasma proteins in brook trout, Salvelinus fontinalis, using precipitation, dialysis, and electrophoretic methods. J. Fish. Res. Bd. Canada, 30: 1131-1140.

Feeny, R.E., and W.D. Brown. 1974. Plasma proteins in fishes. In Chemical Zoology Vol. VIII Edited by M. Florkin and B.T. Scheer. Academic Press, New York and London, pp. 307-329.

Fletcher, G.L. 1975. The effects of capture "stress" and storage of whole blood on the red blood cells, plasma proteins, glucose, and electrolytes of the winter flounder (Pseudopleuronectes americanus). Can. J. Zool. 53: 197-206.

Fontaine, M., and Y.-A. Fontaine. 1956. Détermination du pouvoir thyréotrope de l'hypophyse et du milieu intérieur de téléosteens par mesure de la fixation de ^{131}I par la thyroids de la truite arc-en-ciel (Salmo gairdneri Rich.) J. Physiol. (Paris), 48: 881-892.

Fore, W., P. Kohler, and H. Wynn. 1966. Rapid redistribution of serum thyroxine during ether anaesthesia. J. Clin. Endocrinol. 26: 821-830.

Fryer, J.N. 1975. Stress and adrenocorticoid dynamics in the goldfish, Carassius auratus. Can. J. Zool. 53: 1012-1020.

Fukuda, H., and M.A. Greer. 1975. The effect of basal hypothalamic deafferentation on the nycothermal rhythm of plasma TSH Endocrinology 97: 749-752.

Fukuda, H., M.A. Green L. Roberts, C.F. Allen, B. Critchlow, and M. Wilson. 1975a. Nyctohemeral and sex-related variations in plasma thyrotropin, thyroxine and triiodothyronine. Endocrinology, 97: 1424-1431.

Fukuda, H., N. Yasuda, M.A. Greer, M. Kutas, and S.E. Greer. 1975b. Changes in plasma thyroxine, triiodothyronine, and TSH during adaptation to iodine deficiency in the rat. Endocrinology, 97: 307-314.

Garcia, L.E., and A.H. Meier. 1973. Daily rhythms in concentration of plasma cortisol in male and female gulf killifish, (Fundulus grandis). Biol. Bull. 144: 471-479.

Grandesso , R., P. Pianca, A. DaTos, and R. DeToni. 1974. Variazioni nictemerali della teroxina e dell'indice di saturazione della triiodotironina in soggetti normali. Minerva Medica, 65: 2513-2520.

Gregerman, R.I., 1963. Estimation of thyroxine secretion rate in the rat by the radioactive thyroxine turnover technique: Influences of age, sex and exposure to cold. Endocrinology, 72: 382-392.

Gregory, L.A., and J.G. Eales. 1975. Factors contributing to high levels of plasma iodide in brook trout, Salvelinus fontinalis (Mitchill). Can. J. Zool. 53: 267-277.

Grossie, J., and C.W. Turner. 1962. Thyroxine secretion rates during food restriction in rats. Proc. Soc. Exp. Biol. Med. 110: 631-633.

Hane, S., O.H. Robertson, B.C. Wexler, and M.A. Krupp. 1966. Adrenocortical response to stress and ACTH in Pacific salmon (Oncorhynchus tshawytscha) and steelhead trout at successive stages in the sexual cycle. Endocrinology, 78: 791-800.

Harland, W.A., P.W. Horton, R. Strang, B. Fitzgerald, J.R. Richards, and K.B. Holloway. 1974. Release of thyroxine from the liver during anaesthesia and surgery. Br. J. Anaesth. 46: 818-820.

Harland, W.A., J.S. Orr, and J.R. Richards. 1972. Increased thyroxine secretion following surgical operation. Scott. Med. J. 17: 92-97.

Henderson, N.E. 1976. Thyroxine concentrations in plasma of normal and hypophysectomized hagfish, Myxine glutinosa (Cyclostomata). Can. J. Zool. 54: 180-184.

Henderson, N.E. and F.L. Lorscheider. 1975. Thyroxine and protein-bound iodine concentrations in plasma of the Pacific hagfish, Eptatretus stoutii (Cyclostomata). Comp. Biochem. Physiol. 51: 723-726.

Hickman, C.P., Jr. 1962. Influence of environment on the metabolism of iodine in fish. Gen. Comp. Endocrinol. Suppl. 1: 48-62.

Higgs, D.A. 1974. Influence of nutritional state on thyroid hormone metabolism in the brook trout, Salvelinus fontinalis (Mitchill). Ph.D. Thesis, University of Manitoba.

Higgs, D.A., and J.G. Eales. 1973. Measurement of circulating thyroxine in several freshwater teleosts by competitive binding analysis. Can. J. Zool. 51: 49-53.

Hoar, W.S., and J.G. Eales. 1963. The thyroid gland and low temperature resistance of goldfish. Can. J. Zool. 41: 653-669.

Horth, C.E., A.C. Craig, J.R. Shelton, R.F. Palmer, and M.J. Tidd. 1975. Circadian blood follicle - stimulating hormone, lutinizing hormone, thyroxine, cortisol and aldosterone levels measured simultaneously in man. J. Endocrinol. 64: P. 56.

Houston, A.H., J.A. Madden, R.J. Woods, and H.M. Miles. 1971. Some physiological effects of handling and tricaine methane - sulphonate anaesthetization upon brook trout, Salvelinus fontinalis. J. Fish. Res. Bd. Canada, 28: 625-633.

Houston, A.H., J.T. Corlett, and R.J. Woods. 1976. Specimen weight and MS222. J. Fish. Res. Bd. Canada, 33: 1403-1407.

Howorth, P.J.N., and P. Marsden. 1976. Rapid radioimmunoassay of triiodothyronine on Sephadex G-25 by the Ames Kit. J. Nuclear Med. 17: 321-322.

Huang, C.T., and C.P. Hickman, Jr. 1968. Binding of inorganic iodide to the plasma proteins of teleost fishes. J. Fish. Res. Cd. Canada, 25: 1651-1666.

Ingbar, D.H., and V.A. Galton. 1975. The effect of food deprivation on the peripheral metabolism of thyroxine in rats. Endocrinology, 96: 1525-1532.

Ingbar, S.H., and K.A. Woeber. 1974. The thyroid gland. In Textbook of endocrinology. Edited by R.H. Williams. W.B. Saunders Co. Philadelphia, London, Toronto, pp. 95-232.

Ingemble, Y., P. Denajer, and M. Denisch. 1974. Thyroxine-binding-globulin in infant protein calorie malnutrition. J. Clin. Endocrinol. Metab. 39: 178-180.

Ingenbleek, Y., and C. Beckers. 1975. Triiodothyronine and thyroid stimulating hormone in protein-calorie malnutrition in infants. Lancet, 2: 845-848.

Jacoby, G.H., and C.P. Hickman, Jr. 1966. A study of the circulating iodocompounds of rainbow trout, Salmo gairdneri by the method of isotopic equilibrium. Gen. and Comp. Endocrinol. 7: 245-254.

Johns, M.W., J.P. Masterton, J.E. Paddle-Ledineck, Y.C. Patel, D. Winikoff, and M. Malink. 1975. Variations in thyroid function and sleep in healthy young men. Clin. Sci. Mol. Med. 49: 629-632.

Kirby, R., F. Clark, and I.D.A. Johnson. 1973. The effect of surgical operation of moderate severity on thyroid function. Clin. Endocrinol. 2: 89-92.

Koch, B., M. Jobin, S. Deilai, and C. Fortier. 1972. Thyrotropin (TSH) response to synthetic TSH releasing factor, following pharmacological blockage of adreno-corticotropin (ACTH) secretion. Can. J. Physiol. Pharm. 50: 360-363.

Krebietke, P. 1975a. Diurnal changes of thyroid hormones in cockerel (Gallus domesticus). II. Investigation of protein bound radioiodine and free hormone. Endokrinologie, 65: 42-52.

Krebietke, P. 1975b. Diurnal changes of thyroid hormones in cockerel (Gallus domesticus). III. Investigation of changes in deiodination of thyroxine-I^{131}. Endokrinologie, 65: 53-59.

Kumar, R.E., B.U. Musa, W.G. Appleton, and J.T. Dowling. 1968. Effect of prednisone on thyroxine distribution. J. Clin. Endocrinol. Metab. 28: 1335-1340.

LaRoche, G., C.K. Johnson, and A.N. Woodall. 1965. Thyroid function in the rainbow trout (Salmo gairdneri, Rich). I. Biochemical and histological evidence of radiothyroidectomy. Gen. Comp. Endocrinol. 5: 145-159.

Leatherland, J.F., and B.A. McKeown. 1973. Circadian rhythym in plasma levels of prolactin in goldfish, Carassius auratus L. J. Interdispl. Cycle. Res. 4: 137-143.

Leloup, J., and M. Fontaine. 1960. Iodine metabolism in lower vertebrates. Ann. N.Y. Acad. Sci. 86: 316-353.

Leloup, J., et A. Hardy. 1976. Hormones thyroidiennes circulantes chez un cyclostome et des poissons. Gen. Comp. Endocrinol. 29: 258.

Lemarchand-Béraud, T., and A. Vanottii. 1969. Relationships between blood thyrotropin level, protein-bound iodine and free thyroxine concentrations in man under normal physiological conditions. Acta Endocrinol. 60: 315-326.

Lennon, H.P., and J.P. Mixner. 1957. Variation in plasma protein-bound iodine levels in dairy cattle due to animal, daily, diurnal, and method effects. J. Dairy Sci. 40: 541-545.

Lewis, M., and J.M. Dodd. 1976. Thyroid hormone levels in normal and experimental dogfish (Scyliorhinus canicula). Gen. Comp. Endocrinol. 29: 258.

Love, R.M. 1970. The chemical biology of fishes. Academic Press, London and New York, 547p.

Magwood, S.G.A., and O. Heroux. 1968. Fecal excretion of thyroxine in warm and cold-acclimated rats. Can. J. Physiol. Pharmacol. 46: 601-607.

Margolese, M.S., and O.J. Golub. 1957. Daily fluctuation of serum protein bound iodine level. J. Clin. Endocrinol. 17: 849-853.

Matty, A.J. 1960. Thyroid cycles in fish. Symp. Zool. Soc. Long No. 2, 1-15.

Matty, A.J., and C.C. Thornburn. 1970. Quantitative determination of iodoamino acids in the thyroid gland of the parrot-fish (Scarus guacamaia). J. Endocrinol. 46: 417-423.

McBride, J.R. 1967. Effects of feeding on the thyroid, kidney, and pancreas in sexually ripening adult sockeye salmon (Oncorhynchus nerka). J. Fish. Res. Bd. Canada, 24: 67-75.

Melander, A. 1970. Studies on the thyroid activity in the mouse 2. The effects of stress and changes in corticotropin secretion. Acta Endocrinol. 64: 569-576.

Merimee, T.J., and E.S. Fineberg. 1976. Starvation induced alterations of circulating thyroid hormone concentration in man. Metabolism, 25: 79-83.

Midgley, A.R., G.D. Niswender, and R.W. Regar. 1969. Principles for assessment of the reliability of radioimmunoassay methods (Precision, accuracy, sensitivity, specificity). Acta. Endocrinol. 63, Suppl. 142: 163-180

Mühlen, A.V., M. Lammers, J. Köbberli, and M.Hersch. 1974. TSH corticosterone, and T-3 in rats under various endogenous and environmental conditions. Acta Endocrinol. 75, Suppl. 184: P 128.

Nathanielsz, P.W. 1969. A circadian rhythm in the disappearance of thyroxine from the blood in the calf and the thyroidectomized rat. J. Physiol. 204: 79-90.

Nathanielsz, P.W. 1970. The effect of diet and acute starvation on the deiodination of thyroxine and triiodothyronine in the thyroidectomized rat. J. Physiol. 206: 701-710.

Nejad, I., J. Bollinger, M.A. Mitnick, P. Sullivan, and S. Reichlin. 1975. Measurement of plasma and tissue triiodothyronine concentration in the rat by radioimmunoassay. Endocrinology, 96: 773-780.

Newcomer, W.S. 1974. Diurnal rhythms of thyroid function in chicks. Gen. Comp. Endocrinol. 24: 65-73.

Nicoloff, J.T., D.A. Fisher, and M.D. Appleman. 1970. The role of glucocorticoids in the regulation of thyroid function in man. J. Clin. Invest. 49: 1922-1929.

Nihei, N.N., M.C. Gershengorn, T. Mitsuma, L.R. Stringham, A. Cordy, B. Kuchmy, and C.S. Hollender. 1971. Measurements of triiodothyronine and thyroxine in human serum by gas-liquid chromatography. Anal. Biochem. 43: 433-445.

Nye, L., M. Hassan, E. Willmott, and J. Landon. 1976. Introduction of a rapid, simple radioimmunoassay and quality control scheme for thyroxine. J. Clin. Path. 29: 452-457.

O'Connor, J.F., G.Y. Wu, T.F. Gallagher, and L. Hellman. 1974. The 24-hour plasma thyroxine profile in normal man. J. Clin. Endocrinol. Metab. 39: 765-711.

Odell, W.D., J.F. Wilber, and R.D. Utiger. 1967. Studies of thyrotropin physiology by means of radioimmunoassay. Recent. Prog. Horm. Res. 23: 47-78.

Ohtake, M., and G.A. Bray. 1975. Anaesthetic agents depress release of TRH. Clin. Res. 23: 95A.

Ortman, R., and R.D. Billig. 1966. A re-examination of the goldfish microhistometric method for thyrotropin. Gen. Comp. Endocrinol. 6: 362-370.

Osborn, R.H., and T.H. Simpson. 1972. Iodoamino acids of plaice plasma; the influence of TSH, stress and starvation. Gen. Comp. Endocrinol. 18: 613.

Oyama, T., S. Shibata, A. Matsuki, and T. Kudo. 1969a. Thyroxine distribution during holothane anaesthesia in man. Anesth-Analg. (Clev.), 48: 715-719.

Oyama, T., S. Shibata, A. Matsuki, and T. Kudo. 1969b. Serum endogenous thyroxine levels in man during anaesthesia and surgery. Br. J. Anaesth. 41: 103-108.

Packard, G.C., M.J. Packard, and A. Gorbman. 1976. Serum thyroxine concentrations in the Pacific hagfish and lamprey and in the leopard frog. Gen. Comp. Endocrinol. 28: 365-367.

Pain, R.W., and P.J. Phillips. 1976. Thyroid hormone levels in protein-calorie malnutrition. Lancet, 1: 202.

Pickford, G.E. and J.W Atz. 1957. The physiology of the pituitary gland of fishes. N.Y. Zool. Soc. N.Y. 613p.

Pipes, G.W., J.A. Grossie, and C.W. Turner. 1960. Effect of underfeeding on thyroxine secretion rate of female mice. Proc. Soc. Exp. Biol. Med. 104: 491-492.

Pittman, C.S., J.B. Chambers, Jr., and W.H. Read. 1971. The extrathyroidal conversion rate of thyroxine to triiodothyronine in normal man. J. Clin. Invest. 50: 1187-1196.

Portnay, G.I., J.T. O'Brian, J. Bush, A.G. Vagenakis, F. Azizi, R.A. Arky, S.H. Ingbar, and L.E. Braverman. 1974. The effect of starvation on the concentration and binding of thyroxine and triiodothyronine in serum and on the response of TRH. J. Clin. Endocrinol. Metab. 39: 191-194.

Rabinovitch, J. 1929. The effect of underfeeding on the proliferative activity of the thyroid gland in the guinea pig. Amer. J. Pathol. 5: 87-90.

Rapp, J.P., and L.L. Pyun. 1974. A sex difference in plasma thyroxine and thyroid stimulating hormone in rats. Proc. Soc. Expt. Biol. Med. 146: 1021-1023.

Rastogi, G.K., R.C. Sawhney, N.C. Panda, and B.B. Tripathy. 1974. Thyroid hormone levels in adult protein calorie malnutrition (PCM). Horm. Metab. Res. 6: 528-529.

Refetoff, S., N.I. Robin, and V.S. Fang. 1970. Parameters of thyroid function in serum of 16 selected vertebrate species: a study of PBI, serum T_4, free T_4, and the pattern of T_4 and T_3 binding to serum proteins. Endocrinology, 86: 793-805.

Robertson, O.H., M.A. Krupp, C.B. Fanorer, S. Hane, and S.F. Thomas. 1961. Physiological changes occurring in the blood of the Pacific salmon (Oncorhynchus tschawytscha) accompanying sexual maturation and spawning. Endocrinology, 68: 733-746.

Sadovsky, R., and A.Bensadouin. 1971. Thyroid iodohormones in the plasma of the rooster (Gallus domesticus). Gen. Comp. Endocrinol. 17: 268-274.

Schatz, D.L., R.H. Sheppard, and H.C. Palter. 1967. Thyroid function studies in fasting obese subjects. Metabolism, 16: 1075-1085.

Schatz, D.P., and R. Volpe. 1959. Lack of diurnal variation in the level of serum protein bound iodine. J. Clin. Endocrinol. Metab. 19: 1495-1497.

Seligson, H., and D. Seligson. 1972. Measurement of thyroxine by competitive protein binding. Clin. Chim. Acta, 38: 199-205.

Shambaugh, G.E., and J.F. Wilber. 1974. The effect of calorie deprivation upon thyroid in neonatal rat. Endocrinology, 94: 1145-1149.

Shimizu, T., Y. Shishiba, and S. Yoshimura. 1973. A new radioimmunoassay method for serum triiodothyronine using Sephadex G-25 column. Endocrinol. Japon. 20: 365-268.

Skelley, D.S., L.P. Brown, and P.K. Besch. 1973. Radioimmunoassay. Clin. Chem. 19: 146-185.

Snedecor, G.W., and W.G. Cochran. 1971. Statistical methods. Iowa State University Press, Ames Iowa , 593p.

Sokal, R.R., and F.J. Rohlf. 1969. Biometry. W.H. Freeman and Co., San Francisco, 776p.

Southwood, T.R.E. 1971. Ecological methods. Chapman and Hall, London, 391p.

Spieler, R.E. 1974. Short-term serum cortisol concentrations in goldfish (Carassius auratus) subjected to serial sampling and restraint. J. Fish. Res. Bd. Canada, 31: 1240-1242.

Spieler, R.E., and A.H. Meier. 1976. Short-term serum prolactin concentrations in goldfish (Carassius auratus) subjected to serial sampling and restraint. J. Fish. Res. Bd. Canada, 33: 183-186.

Stephens, D.J. 1940. The effect of the thyrotropic principle of the anterior pituitary on the thyroid of the undernourished guinea pig. Endocrinology, 26: 485-492.

Sterling, K. 1970. The significance of circulating triiodothyronine. Rec. Progr. Horm. Res. 26: 249-286.

Sterling, K., M.A. Brenner, and V.F. Saldanha. 1973. Conversion of thyroxine to triiodothyronine by cultured human cells. Science, 179: 1000-1001.

Sterling, K., and P.O. Milch. 1974. Thermal inactivation of thyroxine-binding globulin for direct radioimmunoassay of triiodothyronine in serum. J. Clin. Endocrinol. Metab. 38: 866-875.

Stevens, E.D. 1968. The effect of exercise on distribution of blood to various organs in rainbow trout. Comp. Biochem. Physiol. 25: 615-625.

Stirling, G.A. 1962. The thyroid in malnutrition. Arch. Dis. Child. 37: 99-102.

Surks, M.I., A.R. Schadlow, and J.H. Oppenheimer. 1972. A new radioimmunoassay for plasma L-triiodothyronine: Measurements in thyroid disease and in patients maintained on hormonal replacement. J. Clin. Invest. 51: 3104-3113.

Talwalker, S.C., and B.B. Gaitonde. 1974. Thyroid function in protein malnutrition. Ind. J. Med. Res. 62: 354-361.

Thurston, R.V. 1967. Electrophoretic patterns of blood serum proteins for rainbow trout (Salmo gairdneri). J. Fish. Res. Bd. Canada, 24: 2169-2188.

Tingley, J.O., A.W. Morris, and S.R. Hill. 1958. Studies of diurnal variation and response to emotional stress of the thyroid gland. Clin. Res. Proc. 1: 134.

Vagenakis, A.G., A. Burger, G.I. Portnay, M. Rudolph , J.T. O'Brian, F. Azizi, R.A. Arky, P. Nicod, S.H. Ingbar, and L.E. Braverman. 1975. Diversion of peripheral thyroxine metabolism from activating to inactivating pathways during complete fasting. J. Clin. Endocrinol. Metab. 41: 191-194.

Van Cauter, E.R., L. Leclerq, L. Vanhaelst, and J. Goldstein. 1974. Simultaneous study of cortisol and TSH variations in normal subjects and patients with hyperadrenalcorticism. J. Clin. Endocrinol. Metab. 39: 645-652.

Vernikos-Danellis, J., C.S. Leach, C.M. Winget, P.C. Ramkaut, and P.B. Mack. 1972. Thyroid and adrenal cortical rhythmicity during bed rest. J. Appl. Physiol. 33: 644:648.

Vilter, V. 1944. Comportement de la thyroide dans la metamorphose de la civelle d'Anguille. C.R. Soc. Biol. 138: 615-616.

Vinik, A., W. Kalk, J. McLaren, S. Hendris, and B.L. Primstone. 1975. Fasting blunts TSH response to synthetic thyrotropin releasing hormone (TRH). J. Clin. Endocrinol. Metab. 40: 509-511.

Walfish, R.G., A. Britton, P.H. Melville, and C. Ezrine. 1961. A diurnal pattern in the rate of disappearance of I^{131} labeled L-thyroxine from the serum. J. Clin. Endocrinol. Metab. 21: 582-586.

Walser, A., H. Luthy, and H.R. Jenzen. 1963. Studies on diurnal variation in the thyroid activity. Acta Endocrinol. Suppl. 67: 99A.

Watson, D., and S. Lees. 1973. Comparative study of thyroxine assays employing kit radio-ligand reagents. Ann. Clin. Biochem. 10: 14-22.

Webster, B.A., A.R. Guansing, and C.J. Paice. 1972. Absence of diurnal variation of serum TSH. J. Clin. Endocrinol. Metab. 34: 899-901.

Wedemeyer, G. 1969. Stress-induced ascorbic acid depletion and cortisol production in two salmonid fishes. Comp. Biochem. Physiol. 29: 1247-1251.

Wedemeyer, G. 1970. Stress of anaesthesia with MS 222 and benzocaine in rainbow trout (Salmo gairdneri). J. Fish. Res. Bd. Canada,27: 909-914.

Weinberg, S.R., J. LoBue, C.D. Siegel, and A.S. Gordon. 1976. Hematopoiesis of the kissing gourami (Helostoma temmincki). Effects of starvation, bleeding, and plasma-stimulating factors on its erythropoiesis. Can. J. Zool. 54: 1115-1127.

Wilber, J.F., and R.D. Utiger. 1967. Immunoassay studies of thyrotropin in rat pituitary glands and serum. Endocrinology, 81: 145-151.

Woods, J.W., J. Wayt, and H.J. Baker. 1966. Cyclic fluctuations in I^{131} content of thyroid glands of cats and monkeys. Proc. Soc. Expt. Biol. Med. 122: 211-214.

Yousef, M.K., and H.D. Johnson. 1966. Does a diurnal rhythm of thyroxine activity exist in dairy cattle? J. Dairy Sci. 49: 209-211.

Zettner, A. 1973. Principles of competitive binding assays (saturation analyses). I. Equilibrium techniques. Clin. Chem. 19: 699-705.

APPENDIX

Appendix Table 1.

Mean values and standard deviations for plasma T_3 and T_4 of rainbow trout. Fresh samples, and samples stored at -20°C for one or 10 days were analysed.

Fish	T_4 (ng%) ($\bar{X} \pm SD$)			T_3 (ng%) ($\bar{X} \pm SD$)		
	Fresh	1 day	10 days	Fresh	1 day	10 days
1	96 ± 9	93 ± 37	115 ± 34	246 ± 42	277 ± 67	222 ± 30
2	56 ± 8	74 ± 24	83 ± 11	190 ± 12	190 ± 7	186 ± 15
3	251 ± 64	283 ± 16	252 ± 21	199 ± 52	254 ± 29	191 ± 15
4	88 ± 3	105 ± 7	84 ± 12	208 ± 12	205 ± 7	179 ± 2
5	185 ± 21	193 ± 16	124 ± 51	373 ± 10	343 ± 46	334 ± 17
6	178 ± 30	234 ± 22	220 ± 72	348 ± 32	371 ± 4	306 ± 28

Appendix Table 2.

Two-way ANOVA comparing plasma T_4 and T_3 between fresh trout plasma, plasma stored one day at -20°C and plasma stored 10 days at -20°C.

Statistical Tests	T_4		T_3	
Bartlett's				
X^2	20.95		30.02	
Significance (P>.01, df=17)	NS		NS	
Two-Way ANOVA				
Total sum squares	280003		271241	
Treatment sum squares	8817		8560	
Row (fish) sum squares	244848		155909	
Interaction sum squares	3572		6329	
Error sum squares	22766		46443	
df total	35		35	
df treatments	2		2	
df rows	5		5	
df interaction	10		10	
df error	18		18	
Mean square treatments	4409		4280	
Mean square rows	48970		31181	
Mean square interactions	357		633	
Mean square error	1265		2580	
F-value treatments	3.48	(NS)	1.66	(NS)
F-value rows	38.72	**	12.09	**
F-value interaction	0.28	(NS)	0.25	(NS)

** Level of significance 0.01

Appendix Table 3.

Number of fish (n), mean ($\bar{X}$) plasma T_4, T_3 and T_4/T_3 with 95% confidence intervals for rainbow trout sampled at various times over two days. Mean weight and standard error (SEM) are also given for each group.

		Weight		T_4			T_3			T_4/T_3		
	Time	$\bar{X}$(g)	SEM	n	$\bar{X}$(ng%)	95%	n	$\bar{X}$(ng%)	95%	n	$\bar{X}$	95%
Day 1	0500	23	1.4	12	179	89-269	12	116	86-156	11	0.84	0.51-1.39
	0700	27	1.7	12	150	99-201	11	151	96-237	11	0.68	0.42-1.12
	1030	25	1.4	12	246	150-341	12	105	81-136	12	1.55	0.99-2.41
	1700	30	3.2	10	215	134-295	10	163	132-202	10	1.06	0.76-1.49
	2230	27	3.2	10	160	90-230	9	113	89-141	9	0.96	0.52-1.80
Day 2	0200	26	2.1	11	217	139-295	11	144	119-175	11	1.03	0.68-1.55
	1030	25	1.8	11	203	151-255	11	188	100-191	11	1.13	0.74-1.74
	1400	27	1.8	11	180	120-240	11	159	122-209	11	0.82	0.52-1.30
	1900	26	1.3	10	256	179-333	10	178	129-244	10	1.15	0.69-1.92
	2230	25	1.8	10	210	156-264	10	146	106-203	10	1.15	0.72-1.82

Appendix Table 4.

One-way ANOVA for plasma T_4, T_3, T_4/T_3 and weight in 10 groups of trout bled at various times over two days.

Statistical Tests	Weight	T_4	T_3[1]	T_4/T_3[1]
Bartletts				
X^2	16.10	10.76	12.38	3.15
Significance (P >.01,df = 9)	NS	NS	NS	NS
One-Way ANOVA				
Total sum of squares	4771.132	1290331.76	3.9993	9.3412
Treatment sum of squares	365.095	111001.41	0.5492	1.0036
Error sum of squares	4406.037	1179330.35	3.4501	8.3376
df total	108	108	106	105
df treatments	9	9	9	9
df error	99	99	97	96
Mean square treatments	40.566	12333.49	0.0610	0.1115
Mean square error	44.505	11912.43	0.0356	0.0869
F	0.91	1.04	1.70	1.28
Significance (P <.05)	NS	NS	NS	NS

[1] Data transformed to $\log_{10}$ to reduce heterogeneity of variance of untransformed data.

Appendix Table 5.

Covariance analysis of regression lines for the correlation of ln plasma T_4 and body weight in male and female rainbow trout.

Group	Means x	Means y	Standard Deviation x	Standard Deviation y	Regression a	Regression b	Correlation Coefficient	F Value	df
♂	26.3	5.110	7.5	0.710	4.339	0.0293	0.309	5.70*	1, 54
♀	25.5	5.149	5.7	0.573	4.388	0.0307	0.295	5.26*	1, 51
Combined	25.9	5.129	6.6	0.643	4.372	0.0292	0.301	10.68**	1, 107

Sum of Products

Source	df	XSS	XYSP	YSS	Red. YSS Due to Reg.	Deviations from Regression df	Deviations from Regression SS	Deviations from Regression MS
Within								
♂	55	3083.74	90.29	27.69	2.644	54	25.0464	0.4638
♀	52	1688.85	51.89	17.05	1.594	51	15.4557	0.3031
Pooled W	107	4772.59	142.18	44.74	4.236	105	40.5021	0.3857
						106	40.5043	0.3821
				Differences between slopes		1	0.0022	0.0022
Between B	1	-1.46	-2.98	-0.01				
W&B	108	4771.13	139.13	44.73	4.061	107	40.6688	0.3801
				Between adjusted means		1	0.1645	0.1645

Tests: 1) Homogeneity of within group variances
F = 1.53 (df = 53, 50) NS

2) Comparison of slopes
F = 0.06 (df = 1,105) NS

3) Comparison of elevations
F = 0.43 (df = 1,106) NS

* Level of significance 0.05
** Level of significance 0.01

Appendix Table 6.

Covariance analysis of regression lines for the correlation of ln plasma T_3 and body weight in male and female rainbow trout.

Group	Means x	Means y	Standard Deviation x	Standard Deviation y	Regression a	Regression b	Correlation Coefficient	F Value	df
♂	26.4	4.994	7.5	0.447	4.334	0.0246	0.421	11.05**	1, 53
♀	25.6	4.863	5.7	0.442	3.854	0.0394	0.505	17.13**	1, 50
Combined	26.0	4.930	6.7	0.447	4.137	0.0305	0.455	27.39**	1, 105

Sum of Products

Source	df	XSS	XYSP	YSS	Red. YSS Due to Reg.	Deviations from Regression df	SS	MS
Within								
♂	54	3058.97	75.39	10.77	1.8580	53	8.9120	0.1682
♀	51	1637.64	64.54	9.97	2.5435	50	7.4265	0.1485
						103	16.3385	0.1586
Pooled W	105	4696.61	139.93	20.74	4.1691	104	16.5709	0.1593
				Differences between slopes		1	0.2324	0.2324
Between B	1	17.59	3.87	0.46				
W & B	106	4714.50	143.80	21.20	4.3861	105	16.8139	0.1601
				Between Adjusted Means		1	.2429	0.2429

Tests: 1) Homogeneity of within group variances
F = 1.13 (df = 53, 50) NS

2) Comparison of slopes
F = 1.467 (df = 1, 103) NS

3) Comparison of elevations
F = 1.523 (df = 1,104) NS

** Level of significance 0.01

Appendix Table 7.

Covariance analysis between regression lines for ln plasma T_4 and T_3 and body weight in trout.

Group	Means		Standard Deviation		Regression		Correlation Coefficient	F Value	df
	x	y	x	y	a	b			
T_3	26.00	4.930	6.70	0.447	4.137	0.0305	0.455	27.39**	1, 105
T_4	25.90	5.129	6.60	0.643	4.372	0.0292	0.301	10.68**	1, 107
Combined	25.96	5.030	6.64	0.563	4.259	0.0297	0.351	29.91**	1, 214

Sum Products

Source	df	XSS	XYSP	YSS	Red due to Reg	Deviations from Regression		
						df	SS	MS
Within								
T_3	106	4714.50	143.80	21.20	4.386	105	16.8139	0.1601
T_4	108	4771.13	139.20	44.73	4.061	107	40.6688	0.3801
Combined	214	9485.15	281.96	68.06	8.381	213	59.6790	0.2802

Comparison of homogeneity of variances

$F = 2.37$ ** (df = 107, 105)

$\therefore$ variances are not homogeneous and it is invalid to compare regressions

** Level of significance 0.01.

Appendix Table 8.

Two-way ANOVA for plasma T_4, T_3 and T_4/T_3 for rainbow trout from Experiment 3 (injection-stress).

Statistical Tests	T_4 [1]	T_3	T_4/T_3 [1]
Bartletts			
X^2	3.06	7.36	1.99
Significance (P >.01, df=5)	NS	NS	NS
Two-way ANOVA			
Total sum squares	5.4101	88055.2	10.5125
Treatment sum squares	0.0055	686.8	0.0234
Row(time) sum squares	0.7690	12055.4	3.6402
Interaction sum squares	0.2055	315.6	0.0043
Error sum squares	4.4301	74997.2	6.8446
df total	63	59	57
df treatments	1	1	1
df rows	2	2	2
df interaction	2	2	2
df error	58	54	52
Mean square treatments	0.0055	686.8	0.0234
Mean square rows	0.3845	6027.7	1.8201
Mean square interaction	0.1028	157.8	0.0022
Mean square error	0.0764	1388.4	0.1316
F value treatments	0.07 (NS)	0.50 (NS)	0.18 (NS)
F value rows	5.03 **	4.34 *	13.8 **
F value interaction	1.35 (NS)	0.11 (NS)	0.012 NS

[1] Data transformed to $\log_{10}$ to reduce heterogeneity of variance in untransformed data.

* Level of significance 0.05
** Level of significance 0.01

Appendix Table 9.

Mean values ($\bar{X}$), numbers of fish (n) and 95% confidence intervals for plasma T_4, T_3 and T_4/T_3 for combined data from Experiment 3 (injection-stress).

	T_4			T_3			T_4/T_3		
Group	n	$\bar{X}$ (ng %)	95%	n	$\bar{X}$(ng %)	95%	n	$\bar{X}$	95%
Control	20	125	100-156	20	68	45-91	20	1.95	1.35-2.82
2 hr.	22	286	221-370	20	36	23-49	20	8.65	5.82-12.85
6 hr	22	199	140-281	22	70	50-90	20	2.34	1.56-3.50
12 hr	20	154	122-194	18	56	38-74	18	2.95	2.06-4.22

Appendix Table 10.

One-way ANOVA comparing combined data for plasma T_4, plasma T_3, T_4/T_3, and weight from Experiment 3 (injection-stress)

Statistical Tests	Weight	T_4 [1]	T_3	T_4/T_3 [1]
Bartletts				
X^2	10.18	6.65	7.49	0.67
Significance (df=3, P >.01)	NS	NS	NS	NS
One-way ANOVA				
Total sum squares	1589.51	6.9895	137095.39	14.0885
Treatment sum squares	29.01	1.5118	14067.96	5.0236
Error sum squares	1560.51	5.4777	123027.43	9.0649
df total	83	83	79	77
df treatment	3	3	3	3
df error	80	80	76	74
Mean square treatment	9.669	0.5039	4689.32	1.6745
Mean square error	19.506	0.0685	1618.78	0.1225
F value	0.50	7.36	2.90	13.67
Significance	NS	**	*	**

[1] Data transformed to $\log_{10}$ to reduce heterogeneity of variance in untransformed data.

* Level of significance 0.05
** Level of significance 0.01

Appendix Table 11.

Newman-Keuls test to locate differences between means of combined data from Experiment 3 (injection-stress).

1. T_4 (data transformed by $\log_{10}$ to reduce heterogeneity)

RANKED MEANS:

	Control	12 hr	6 hr	2 hr
n	20	20	22	22
$\overline{X}$	2.0963	2.1879	2.2981	2.4567
S	0.2061	0.2145	0.3394	0.2519

pooled variance = 0.2606

SUMMARIZED DIFFERENCES:

Control	12 hr	6 hr	2 hr

2. T_3

RANKED MEANS:

	2 hr	12 hr	Control	6 hr
n	20	18	20	22
$\overline{X}$	35.8	56.2	67.9	69.6
S	26.8	35.2	48.9	44.3

pooled variance = 39.96

SUMMARIZED DIFFERENCES:

2 hr	12 hr	Control	6 hr

Appendix Table 11 (continued)

3. T_4/T_3 (data transformed by $\log_{10}$ to reduce heterogeneity)

RANKED MEANS:

	Control	6 hr	12 hr	2 hr
n	20	20	18	20
$\overline{X}$	2.2913	2.3693	2.4700	2.9371
S	0.3397	0.3733	0.3133	0.3667

pooled variance = 0.3500

SUMMARIZED DIFFERENCES:

Control	6 hr	12 hr	2 hr

(lines join similar means)

Appendix Table 12

Two-way ANOVA for weight, plasma T_4, plasma T_3 and T_4/T_3 for the groups of trout from Experiment 5 (time of starvation)

Statistical Tests	Weight	T_4	T_3 [1]	T_4/T_3
Bartlett's				
X^2	9.95	6.19	15.74	14.04
Significance (P >.01, df=5)	NS	NS	NS (P>.005)	NS
Two-way ANOVA				
Total sum squares	7178.7	317417.2	1.5469	4.8992
Treatment sum squares	245.2	27660.2	0.1255	0.1359
Row (time) sum squares	295.0	1282.6	0.0452	0.2639
Interaction sum squares	606.5	3965.0	0.4206	0.9969
Error sum squares	6032.0	284509.4	0.9556	3.5038
df total	45	45	43	43
df treatment	1	1	1	1
df rows	2	2	2	2
df interaction	2	2	2	2
df error	40	40	38	38
Mean square treatments	245.2	27660.2	0.1255	0.1359
Mean square rows	147.5	641.3	0.0226	0.1320
Mean square interaction	303.3	1982.5	0.2103	0.4978
Mean square error	150.8	7112.7	0.0245	0.9343
F value treatment	1.63 (NS)	3.89 (NS)	5.12 *	0.15 (NS)
F value rows	0.98 (NS)	0.09 (NS)	0.92 (NS)	0.14 (NS)
F value interaction	2.01 (NS)	0.28 (NS)	8.58 **	0.53 (NS)

[1] Data transformed $\log_{10}$ to reduce heterogeneity of variance in untransformed data.

* Level of significance 0.05

** Level of significance 0.01

Appendix Table 13

Newman-Keuls test to locate differences between means for plasma T_3 for starved and fed fish. The original data were transformed by $\log_{10}$ to reduce heterogeneity.

RANKED MEANS:

	7 day starved	3 day fed	14 day starved	3 day starved	7 day fed	14 day fed
n	7	8	7	8	7	7
$\overline{X}$	2.2328	2.2496	2.2611	2.4058	2.4856	2.5226
S	0.0591	0.1022	0.1583	0.2762	0.1149	0.1274

pooled variance = 0.1553

SUMMARIZED DIFFERENCES:

7 day starved	3 day fed	14 day starved	3 day starved	7 day fed	14 day fed
———————		———————			

(lines join similar means)

Appendix Table 14

Mean ($\bar{X}$) plasma T_4, T_3, T_4/T_3 and mean fish weights with 95% confidence intervals or standard error of the mean (SEM) in fish from Experiment 5 (time of starvation).

Group		Weight		T_4			T_3[1]			T_4/T_3		
Day	Condition	$\bar{X}$(g)	SEM	n	$\bar{X}$(ng%)	95%	n	$\bar{X}$(ng%)	95%	n	$\bar{X}$	95%
3	Fed	40.0	4.2	8	202	150-254	8	178	146-216	8	1.01	0.75-1.27
	Starved	44.4	4.2	8	178	89-267	8	255	151-428	8	0.52	0.43-0.61
7	Fed	50.7	2.6	8	208	140-276	7	306	240-39	7	0.58	0.37-0.79
	Starved	44.7	5.9	8	146	65-227	7	171	151-194	7	0.57	0.36-0.78
14	Fed	53.9	4.7	7	213	114-302	7	333	254-437	7	0.56	0.30-0.82
	Starved	40.5	5.1	7	150	110-190	7	182	130-256	7	0.77	0.28-1.26

[1] Data transformed by $\log_{10}$ to reduce heterogeneity in untransformed data.