Order Number 9209702

Thyroid hormone binding to brain nuclear extracts during smoltification in coho salmon

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THYROID HORMONE BINDING TO BRAIN NUCLEAR EXTRACTS DURING SMOLTIFICATION IN COHO SALMON

A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

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Fairbanks, Alaska

May 1991

THYROID HORMONE BINDING TO BRAIN NUCLEAR EXTRACTS DURING SMOLTIFICATION IN COHO SALMON

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ABSTRACT

Salmon complete a metamorphosis called smoltification prior to entering salt water. Increased thyroid activity, olfactory imprinting, and chemical and structural changes in the brain are known to occur at this time. This study was undertaken to determine if triiodothyronine (T_3) binding to brain nuclear extracts changes during smoltification.

During this investigation serum thyroxine (T_4) concentrations increased three fold during smoltification coincident with changes in coloration and morphology and surged again during downstream migration to six times presmolt concentrations. Using ultrafiltration assays, homologous displacement experiments on KCl extracts of recovered brain cell nuclei indicated that maximal binding capacity increased during smoltification and down-stream migration. The increase in receptor concentration lagged the increase in serum thyroxine by one week. Dissociation constants increased during smolt transformation but declined abruptly during down-stream migration. However, dissociation constants did not change during smoltification if nuclear extracts had been previously incubated at room

temperature to remove endogenous ligand. Dissociation rate increased significantly, coincident with the increase in receptor concentration measured by homologous displacement. The maximal probable percent occupancy of available receptors increased from 60% before to greater than 95% during the smolt transformation climax. These results provide evidence that thyroid hormone receptors participate in brain development and olfactory imprinting in smolting salmon.

Dedication

To

Susan, Ben, and Lucas

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ACKNOWLEDGMENTS

This research was supported by funds supplied by NIH, NSF, the Alaska Sea Grant Program, and private sources. Fish and generous advice were obtained courtesy of Bob Och and Steve Bates of the Alaska Department of Fish and Game, Crooked Creek Hatchery. The attention and guidance by my committee is much appreciated, especially the patience and fortitude of Sven Ebbesson. Don Button's persistent spurs awakened my evolving sense of enzyme kinetics and I enjoyed the grasp of new ideas shared with Dave Stone and Larry Duffy. Other members of the committee, Tom Royer, Bob Eisner, Ron Smith, inspired me with their intellectual energy and discipline. Gerry Plumley and Scott Smiley were always willing to discuss the "next step" and the logical implications of the results. Tsuneo Nishiyama and Vera Alexander deserve special thanks for having faith in me while this idea fledged.

Of all the people who assisted this endeavor, none can compare to the quiet patience of my wife, Susan. Without her financial, moral, and intellectual support, I would never have completed this.

INTRODUCTION

Pacific salmon are a very important economic resource along the North Pacific Rim. They are also biologically fascinating animals with distinct physiological and behavioral changes throughout life. Perpetuation of the resource in the face of growing human populations and industrial development may be dependent on a better understanding of those factors which affect critical periods of the life cycle. I wish to focus on one such period, the transition from fresh water to the oceans, a metamorphosis called smoltification.

Thyroxine concentrations surge during smoltification and participate in the distinctive silvering, down-stream migration, and salt water tolerance that characterize this transition period. Salmon imprint the olfactory characteristics of the natal stream during smoltification. The imprint is retrieved during sexual maturation and guides migration back to the spawning grounds. Olfactory imprinting is dependent on thyroid hormone (TH).

Retinal and olfactory projections and some neurotransmitter systems change substantially during salmon smoltification. The participation of thyroid hormone in these processes is unknown but TH is required for proper neural development in higher vertebrates. Since hormone binding precedes its biological activity, the goal of this investigation was to determine the TH binding affinity and receptor concentration in brain tissue obtained from coho salmon throughout the course of smolt transformation. It was concluded that thyroid hormone receptor concentration increased substantially during smoltification, especially during down-stream migration.

SMOLTIFICATION, HOMING, AND THYROID HORMONE

The homing migration and olfactory imprinting. Pacific salmon migrate continuously after leaving fresh water and may spend one to several years in the ocean, traveling thousands of miles before returning to natal streams to spawn and die. The spawning migration reflects an innate navigational system of remarkable accuracy and is one of nature's more intriguing phenomena. Understanding the mechanisms driving such dependable directionality has excited imaginations for many years (Hasler et al., 1978). Recent reviewers agree that salmon use odor to guide their final migration (Doving et al., 1985; McKeown, 1984; Stabell, 1984).

Electrophysiological evidence indicates that migrating salmon increase neural activity when exposed to water from their home streams (Dizon et al., 1973). The home stream is identified during a relatively brief period because, with some allowances for straying, adult salmon return only to the stream in which they smolted (Brannon and Quinn, 1990; Jensen and Duncan, 1971; Mighell, 1975; Peck, 1970) even though they may have originated in a hatchery elsewhere (Jensen and Duncan, 1971). There does not appear to be a cohort dominated olfactory signal as suggested by some workers (Stabell, 1984) because the fish can remember an artifical odor and follow it during the spawning migration if they had been been exposed to that material during smoltification (Hasler et al., 1978).

The olfactory memory appears to lie dormant during the marine phase because the fish does not respond in any distinctive way to smolt-stream odors. The memory is activated; however, during sexual maturation and guides the fish to the home stream to spawn. (Hasler and Scholz, 1983). Because the exposure period

during smoltification is brief, irreversible, and affects future behavior, the home stream memory has been called an olfactory imprint (Hasler et al., 1978).

Imprinting differs from other conventional forms of learning in that it occurs during an extremely brief sensitive period, is stable and persistent, and selectively affects future behavior (Gottlieb, 1987). Imprinting is probably widespread throughout the animal kingdom but is often obscured by other perceptual learning in the developing animal. Even though the young salmon has been exposed to the olfactory code of its natal stream for several months or years, it does not imprint on that code until it undergoes smolt transformation. Further, the imprint persists throughout life, but does not affect behavior until triggered by a change in reproductive maturity. The relative simplicity of the salmon brain potentially creates an ideal model for the neuroethologist who wishes to understand the neural basis of the signal-response relationship, the releasing mechanism that triggers a specific action, and the effects of shifting motivational states. The olfactory imprint interests the developmental neurologist and biochemist because the same hormones that appear intimately associated with smoltification also affect neural development (Fisher et al., 1982; Hamburgh et al., 1971; Kovacs, 1973; Lauder, 1983; Schapiro, 1968; Seiger and Granholm, 1986; Timeras and Nzekwe, 1989). For instance, increased TH activity may be required for imprinting to occur. Fish artifically (and incompletely) smolted by injections of thyroid stimulating hormone (TSH) will imprint an olfactory signal whereas fish that do not experience increased thyroid activity do not imprint (Hasler and Scholz, 1983).

Smoltification. The term "smoltification" describes a physiological and

metamorphic transformation. Like other metamorphoses, smoltification is a dynamic process involving many changes that occur at different rates, but the physiological agents that regulate these different processes have not been conclusively defined in salmon. The transformation alters a cryptically colored, positively rheotactic, stream-dwelling juvenile called a parr to a silvery colored organism fully capable of growth and survival in salt water. Thus far, four types of changes have been characterized: 1) configuration and coloration, 2) osmoregulation, 3) metabolic and biochemical alterations, and 4) changes in behavior.

Configuration and coloration. The vivid parr marks become masked by the deposition of purines which cause the fish to appear silvery (Hayashi, 1970) and the tips of dorsal and tail fins become pigmented. The condition factor decreases as the fish becomes more streamlined (Gorbman et al., 1982) and the growth rate increases (Folmar and Dickoff, 1981; Vanstone and Markert, 1968). The thickening dermis may contribute to the appearance of palpebrae (Barrington, 1961), cloacal folds and pelvic fin appendages (Gorbman et al., 1982). Fish that fail to completely smolt prior to salt water exposure do not grow normally and "stunt" (Folmar et al., 1982), but may retain some characteristics of smolts if they survive (Gorbman et al., 1982). Smolts that do not enter salt water during a rather narrow time window revert to parr (Woo et al., 1978).

Osmoregulation. The most critical feature of anadromony is the ability to alter osmoregulatory mechanisms maintaining electrolyte homeostasis while the environment changes from hypoosmotic fresh water to hyperosmotic seawater. In the first case ions are retained and water excreted; in the second, salts are excreted and water is conserved. As the fish makes the transition from fresh to salt water,

prolactin, a fresh water adapting hormone (Hirano, 1986), is replaced with growth hormone and cortisol: hormones that promote water retention and salt excretion (Hoar, 1988). The gill becomes a major excretory organ for monovalent ions. Gill NaP^+/K^+ ATPase increases substantially during smoltification (Boeuf and Prunet, 1985). Interestingly, the increase in gill Na^+/K^+ ATPase, kidney tubular resorption, and intestinal fluid absorption occur while the fish is still in freshwater and before down-stream migration (Boeuf and Prunet, 1985; Collie and Bern, 1982; Potts et al., 1970). It has been argued that these changes occur preparatory to marine life (Hoar, 1988), but if the mechanisms for salt excretion and fluid retention were fully active during down-stream migration, the fish would become hyponatremic before reaching the ocean.

When the gonads develop and the fish is ready to return to fresh water to spawn, the ability to osmoregulate in salt water declines. The increased osmotic stress is accompanied by increased cortisol and growth hormone concentrations. Prolactin concentrations do not increase until the fish is in fresh water (Hirano et al., 1990). The inability to maintain physiologic electrolyte concentrations with progressing sexual maturation in the ocean is probably a major force behind the compulsion to return to fresh water.

Metabolic and biochemical differences. Biochemically, the smolt is radically different from the parr that preceded it. Oxidative metabolism increases and is accompanied by depletion of lipid and glycogen reserves (Sheridan, 1985). Serum glucose, fat, and protein are significantly reduced in the fresh water smolt (Woo et al., 1978). Increased synthesis of long chain polyunsaturated fatty acids accompanied by decreased amounts of linoleic acid contribute to marine adaptation

(Sheridan, 1986). The rate of protein influx in the intestine is increased, possibly in response to the bioenergetic demands of increased growth (Collie and Stevens, 1985). Hemoglobin polymorphs change to the adult pattern (Sullivan et al., 1985) and demonstrate a greater affinity for oxygen (Giles and Randall, 1980).

Behavioral changes. As discussed above, olfactory imprinting is a major behavioral feature of smoltification. In addition, the advanced fresh water smolt changes from a territorial, positively rheotactic fish to a schooling, downsteam migrant (Folmar and Dickoff, 1980). The change in stream orientation is sudden, occurring in a matter of hours. In a hatchery situation, the behavior appears initiated by increases in water temperature and results in agitated swimming and severe crowding at the lower end of the raceway. Because of mortalities from local oxygen depletion and injuries, the behavior is used by some hatchery personnel as an indicator of time to release (Bob Och, Manager, Crooked Creek Hatchery, pers. comm.). The change in stream orientation does not occur until the completion of a surge in serum thyroxine concentrations (Zaugg et al., 1983), which may be induced with thyroxine treatment (Godin et al., 1974). Fish released prior to the surge remain in shallow water where positive rheotaxis is reinforced with visual stimuli (Arnold, 1974) and move downstream very slowly. Fish released after the thyroxine surge swim continuously and vigorously downstream, usually at night (Thorpe and Morgan, 1978), and can be seined more frequently from deep, but not shallow water (Zaugg et al., 1983). The negatively rheotactic behavior persists as the fish actively swims with ocean currents throughout most of its marine life. That behavior is not altered until the onset of the spawning migration (Royce et al., 1968).

Endocrine and environmental control of smoltification. In addition to the age and size of the fish, the environment may modulate the onset of smoltification. Photoperiod and temperature serve as environmental periodicities, or zeitgebers, the phase and period of which shift an endogenous circannual rhythmicity (Rusak et al., 1990; Page, 1987). The rate of change of photoperiod appears to entrain the tendency to smolt; increasing day length stimulates smoltification (Saunders et al., 1985; Wagner, 1974; Zaugg and Wagner, 1973; Zaugg, 1981).

Higher temperatures accelerate smoltification but also accelerate the rate of smolt to parr reversion if the fish fails to attain salt water (Wedemeyer et al., 1980) and thereby narrows the time window available for the fish to successfully enter the ocean. Lunar phase may operate as a zeitgeber. High correlations of lunar phase and the thyroxine surge have been reported (Grau, 1982, 1988; Grau et al., 1982, 1985). Down-stream migration of coho smolt have been observed to peak during the new moon (Mason, 1975). Significantly larger adult returns occurred when coho smolts were released from hatcheries during the new moon (Nishioka et al., 1983). However, numerous exceptions exist (Lin et al., 1985; Cheek and Ebbesson, Addendum 1) and smoltification in Salmo does not appear entrained by the lunar cycle (Youngson et al., 1985, 1986). Other environmental factors such as seasonal variations in temperature or stream volume may override any lunar influence.

Pituitary growth hormone and prolactin activities increase during smoltification. Prolactin is important for maintaining hydromineral balance between tissues and the fresh water environment while GH enhances

osmoregulatory capacity and survival in salt water (Hirano, 1986). During amphibian metamorphosis prolactin and TH are antagonistic: prolactin treatment favors retention of larval characteristics (Bern and Nicoll, 1968). Corticosteroids may potentiate salt water tolerance by stimulating Na^+/K^+ATPase (Forrest et al., 1973) and affect lipid metabolism in coho parr but not smolts (Sheridan, 1986). A substantial increase in plasma cortisol occurs following salt water exposure (Specker and Schreck, 1982).

Like amphibian metamorphosis, some smoltification processes may be enhanced or controlled by TH. Thyroid hormone affects silvering (Scholz, 1980), down-stream migratory activity (Zaugg et al., 1983), salinity tolerance (Baggerman, 1963; Folmar and Dickoff, 1980) and is reported required for olfactory imprinting (Hasler and Scholz, 1983). However, observations from our laboratory suggest that fish treated with the antithyroid compound propylthiouracil (Greer et al., 1964) continue to develop as normal appearing smolts even though plasma thyroxine fails to develop the surge characteristic of smoltification (Cheek and Ebbesson, Appendum 1). Secondly, parr treated with TH do not undergo a complete smolt transformation (Eales, 1979; Hasler and Scholz, 1983). Thyroid hormone appears to enhance smolting characteristics regulated by other endocrine factors (Hoar, 1988).

Figure 4 illustrates the large increase in serum thyroxine concentrations that occurs during smoltification. Cortisol concentration meanwhile reaches a maximum during the highly stressful period of osmoregulatory adjustment to salt water (Specker and Schreck, 1982). My observations suggest that two TH surges may occur during smoltification; one occurs during late May in our stock while the

other can sometimes be detected about the time the fish begins down-stream migration.

General features of thyroid hormone synthesis and regulation. Thyroid hormone is an iodinated derivative of tyrosine. A generalized synthetic scheme is illustrated in Figure 1. Iodination occurs in the thyroid gland, the major proteinaceous product of which is the glycoprotein thyroglobulin. Following thyroglobulin synthesis and glycosylation on the endoplasmic reticulum, the protein is transported or diffuses to the apical margin where tyrosyl residues are iodinated, a process which appears to stabilize the protein. Iodinated tyrosyl residues are brought into opposition and an iodophenyl group transferred to form the diphenyl ether linkage, leaving serine as the probable reaction product remaining in the peptide (Robbins et al., 1974). Thyroglobulin is stored in colloid that fuses with lysosomes and subsequently is hydrolyzed releasing tetraiodothyronine or thyroxine (T_4).

Colloid formation and exocytosis is under the influence of the pituitary hormone thyrotropin, also known as thyroid stimulating hormone (TSH), which is under feed-back regulation by triiodothyronine (T_3) (Scanlon and Hall, 1989). TSH is also under direct influence of thyroid releasing hormone (TRH) released into the pituitary portal system from the hypothalamic arcuate and paraventricular nuclei. The hypophyseal-pituitary-thyroid axis is illustrated in Figure 2. The hypothalamic centers respond to environmental factors such as cold (Morley, 1981) or dietary iodide deficency (Bachtarzi and Benmiloud, 1983).

Thyroxine circulates in serum bound to thyroxine binding globulin from which it is released at the target tissue. Peripheral deiodination to triiodothyronine

(T_3) appears the major source of this hormone (Crantz et al., 1982; Surks et al, 1973) although some deiodination also occurs in the thyroid gland (Larsen and Silva, 1983; Robbins et al., 1974). T_3 is considered the active form of the hormone (Larsen and Silva, 1983; Oppenheimer et al., 1974; Surks et al., 1975; Tata and Widnell, 1966;). The source of cellular T_3 may be either local deiodination in the cytoplasm of target tissue (Surks et al., 1975) or exchange with the plasma pool depending on the tissue (Obregon et al., 1979; Surks and Oppenheimer, 1977).

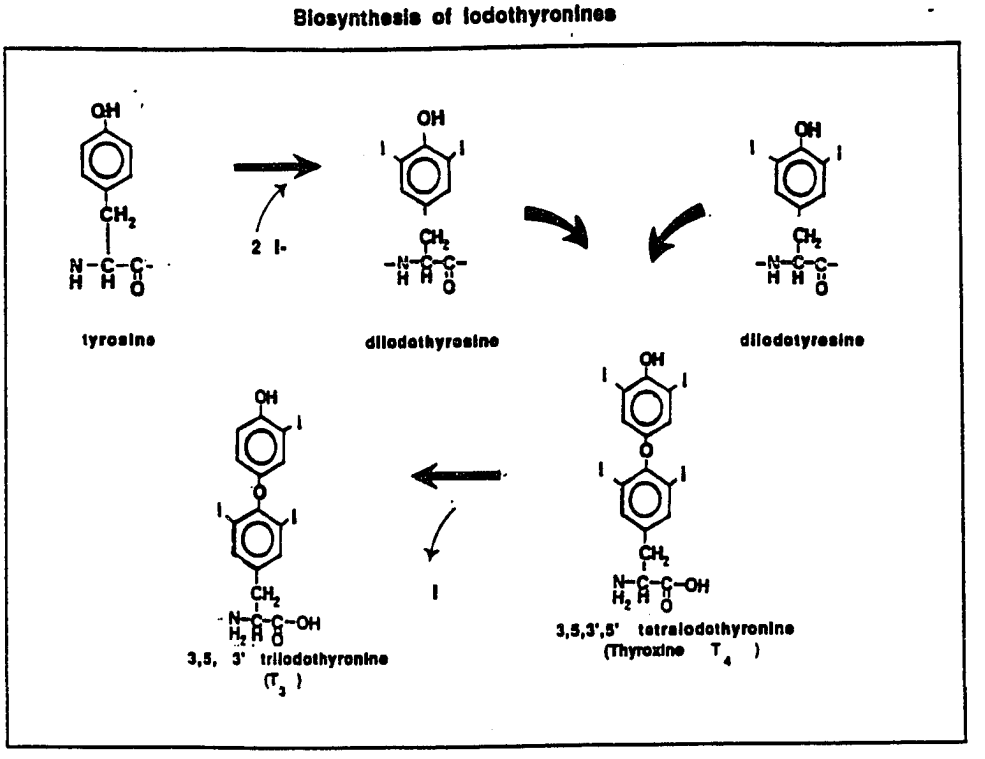


Figure 1. Biosynthetic scheme for iodothyronines in the thyroid gland.

Hypothalamic-pituitary-thyroid control of thyroxine synthesis

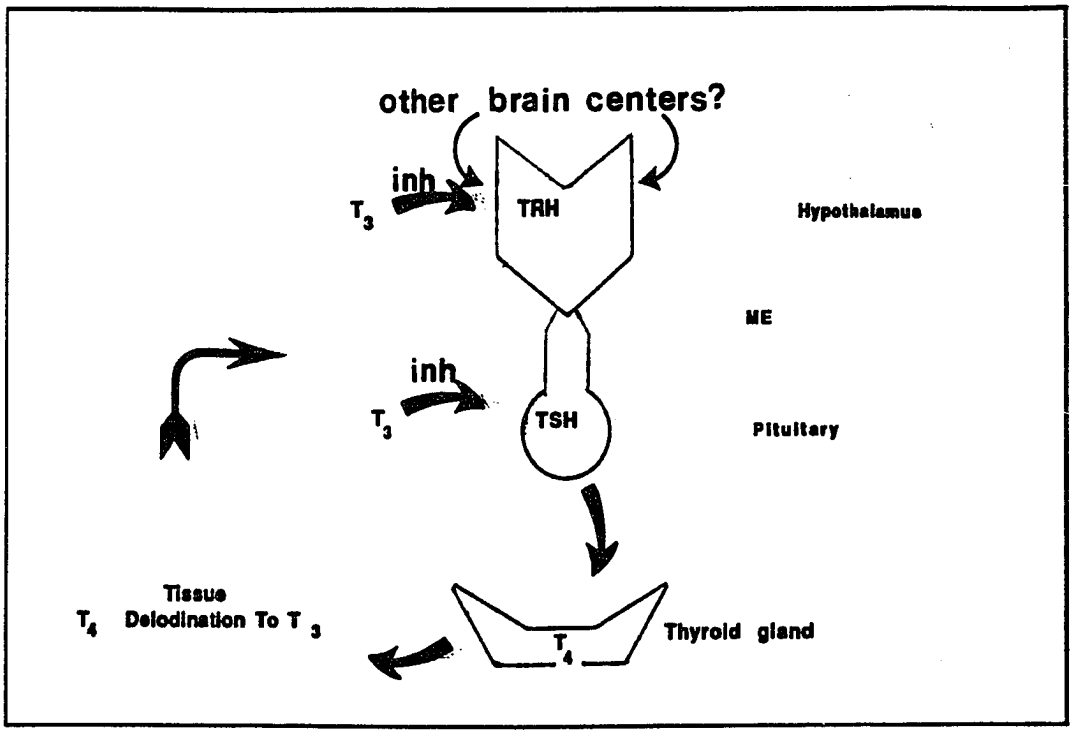


Figure 2. Schematic representation of the control of thyroxine synthesis. Other brain centers include the possible involvement of circadian pacemakers and cortical centers. ME= median eminence, TSH= thyroid stimulating hormone, TRH= thyroid releasing hormone, inh= inhibition.

Thyroid hormone mediated responses. In both the adult and developing animal numerous metabolic processes are influenced by TH including tissue oxygen consumption, calorigenesis, mineral balance, and the synthesis and metabolism of protein, lipid, and carbohydrate (Lavin, 1989). However, the mature brain appears largely unresponsive to TH as measured by oxygen consumption,

mitochrondial alpha glycerol dehydrogenase, and cytosolic malic enzyme activity (Oppenheimer, 1983) . TH stimulated oxygen consumption appears to have both an immediate and a delayed response (Nelson, 1990). TH increases the activity of the Na^+ pump, stimulating Na^+ efflux and the concentration of Na^+/K^+ATPase (Guernsey and Edelman, 1983). Ca^{+2} influx across the plasma membrane is promoted (Segal, 1990). The delayed response appears influenced by TH regulated transcription of metabolic enzymes such as malic acid (Dozin et al., 1986; Strait et al., 1989) and the glucose transporter (Weinstein, 1990).

Glucose absorption, production and utilization is enhanced by TH. Furthermore, TH may potentiate the activity of insulin resulting in increased glucose utilization and glycogen synthesis (Mariash and Oppenheimer, 1983). Protein synthesis and catabolism are stimulated by TH (Goldberg et al., 1980). The enhanced catabolism of protein observed during the hyperthyroid state contributes to the muscle erosion that occurs in thyrotoxicosis.

In the young mammal, TH has profound and widely recognised affects on development and growth. Thyroid hormone stimulates cartilage proliferation and the maturation of bone (Schwartz, 1983). The transformation of hemoglobin to the adult form appears to be under TH control in smolting salmon (Sullivan et al., 1985). In mammals, transfer to extrauterine life is facilitated by TH stimulation of the synthesis of surfactants in the aveoli of the developing lung. Low thyroid activity at birth as in premature infants or hypothyroid individuals may contribute to respiratory stress syndrome (Schwartz, 1983).

Normal growth, differentiation, and development of the nervous system are dependent on TH (Eayrs, 1961). Nerve fiber arborization, synaptogenesis, and

differentiation. Neonatal hypothyroidism can lead to abnormal cerebral electrical activity (Bradley et al., 1960; Lenard and Bell, 1973) and a state of mental retardation called cretinism in man. Prenatal alcohol exposure appears to decrease thyroid function and some of the neurological deficits associated with fetal alcohol syndrome in rats can be partially corrected by TH treatment (Gottesfeld and Silverman, 1990; Hannigan and Bellasario, 1990).

MOLECULAR BASIS OF THYROID HORMONE ACTION

The ligand and the receptor. The product of outer ring deiodination of thyroxine, 3, 5, 3' triiodothyronine (T_3), is the predominate source of TH effects in vivo (Oppenheimer, 1983; Surks and Oppenheimer, 1977). While small amounts of T_3 are released by the thyroid gland, most deiodination occurs in peripheral tissue (Silva and Larsen, 1983). Displacement binding indicates that the prohormone T_4 will occupy the same receptor as T_3 but with significantly less affinity (Oppenheimer et al., 1974).

In vivo response to TH or its analogs is mediated by binding of hormone to a nuclear receptor. After ligand binds to the receptor, chromatin structure is altered (Nyborg and Spindler, 1986) and RNA synthesis increases (Dillman et al., 1978; Lindholm, 1984; Tata and Widnell, 1966). Thyroid hormone nuclear receptors are transcriptional regulators. One type of TH receptor belongs to a family of proteins that includes steroid, retinoic acid, and vitamin D receptors (Evans, 1988). They are products of genes that have a high degree of similarity to the viral oncogene erbA, thus the name erbA genes (Weinberger et al., 1986). The erbA receptors are nuclear proteins. While most steroid receptors are found in the cytoplasm, they generally bind with high affinity to chromatin sites only after modification by hormone binding. For instance, the glucocorticoid receptor is co-translated with a heat shock protein that dissociates subsequent to cytoplasmic ligand binding and confers the receptor competent to bind DNA (Dahlman et al., 1990). Thyroid hormone receptor is an acidic non-histone protein (Surks et al., 1973) that moves into the nucleus and binds to DNA (Jump et al., 1981) soon after translation in the cytoplasm (Dahlman et al., 1990).

Receptor structure and function. Proteolysis of erbA receptors indicates that hormone binding and DNA binding portions occupy different domains. Substitution of the DNA binding domain with that from another hormone receptor results in a switch in template specificity (Green and Chambon, 1987). The centrally located DNA binding portion of the receptor is highly conserved within the erbA family with nine invariant cysteine and one invariant histidine residues. The region folds into two zinc coordinated "fingers" that interact with DNA (Miller et al., 1985). The fingers differ in their hydrophobicity and in the intervening residues which may mediate DNA binding specificity (Green et al., 1988). One finger binds in the major groove of a DNA sequence now known as a TH response element (TRE) (Schlief, 1988) while the other finger appears to stabilize DNA-protein interactions (Green and Chambon, 1987; Green et al., 1988).

The amino terminus is highly variable and subject to casein kinase mediated phosphorylation which may alter the capacity to regulate gene expression (Glineur et al., 1989). The carboxy terminus contains the ligand binding domain and, while variable, shares signficant homology within the erbA family (Weinberger et al., 1986). One highly conserved area is a 20 amino acid sequence which, while not directly involved in ligand binding, appears to directly regulate transcriptional activity. Mutations of this area result in receptors that bind ligand and DNA similarly to the wild type but have impaired transcriptional activity (O'Donnell and Koenig, 1990). Such mutations may account for generalized TH resistence (Sakurai et al., 1989; Usala et al., 1990). Deletion of the carboxy terminus eliminates hormone binding, but the protein remanent containing the DNA binding domain is transcriptionally active, suggesting that the hormone

binding domain modulates transcription (O'Donnell and Koenig, 1990).

Thyroid hormone receptor family. At least five different TH nuclear receptors have been identified (Cook and Koenig, 1990; Nakai et al., 1988; Strait et al., 1990) and appear to be quantitatively tissue specific (Bradley et al., 1989; Macchia et al., 1990; Murray et al., 1988; Thompson, et al., 1987). In both rats and man, the two major groups of TH receptors are derived from different chromosomes (DeGroot et al., 1989). Alternative splicing has been suggested from cDNA sequences for two alpha TH receptors. Another alpha variant is transcribed from the antisense strand and binds, with relatively high affinity (Izumo and Madhavi, 1988), small but measurable amounts of T_3 (Miyajima et al. 1989). The three alpha receptors are present at different times during development (Prost et al., 1988) and expression may be tissue specific (Miyajima et al., 1989). Alpha 1 is a functional receptor but alpha 2, while abundant in the adult brain and very similar to alpha 1, does not bind TH (Mitsuhashi et al.,1988). Yet another variant (alpha 3?) appears to bind hormone but does not promote transcription (Strait et al., 1990). Furthermore, transfection of alpha 2 cDNA can block the effects of co-transfected transcriptionally active alpha 1 and beta 1 gene products (Koenig et al., 1989). The beta 1 gene transcription product is widely distributed in the brain but the beta 2 receptor is confined to the pituitary (Cook and Koenig, 1990)

Thyroid hormone receptor as a transcriptional regulator. The receptor/hormone complex is associated with transcriptionally active chromatin (Nyborg and Spindler, 1986). Hormone binding initiates the synthesis of both ribosomal and mRNA (Dillman et al., 1978; Tata et al., 1963; Tata and Widnell, 1966). Large increases in bacterial RNA polymerase binding capacity occur after

hormone binding, but the increased binding capacity exceeds the number of T_3 receptors in the nuclei. This suggests that each T_3 receptor interaction may initiate the binding of multiple RNA polymerizing enzymes or that hormone binding may reversibly modify chromatin structure (Nyborg and Spindler, 1986; Samuels et al., 1973).

Hormone binding results in the expression or repression of a subset of genes in specific cell types. Genes that are transcriptionally regulated as a result of hormone binding contain short palidromic sequences called hormone response elements (HRE) upstream from the target gene. Thyroid HREs need not share sequence homology (Glass et al., 1987) and therefore may have differing affinities for TR (Degroot et al., 1989).

Modulation of transcriptional activity.

Role of hormone response elements. The interaction of HREs presents enormous capacity for quantitative regulation of transcription. Several HREs, occur upstream from the growth hormone gene (Brent et al., 1989; Crone et al., 1990; Norman et al., 1989) but do not share significant homology. The lack of homology among the different potential HREs suggests that the receptor saturates the available DNA receptor sites with variable affinities (Sap et al., 1990). Both positive and negative HREs occur 5' to the growth hormone gene (Wright et al., 1987). Growth hormone transcription is synergistically activated by glucocorticoids (Martial et al., 1977; Samuels et al., 1977) but is repressed by epidermal growth factor which is also modulated by TH (Walker et al., 1982). Retinoic acid will bind to thyroid HREs (Umesono et al., 1980). It has been shown recently that retinoic acid and TH receptors form heterodimers with different

transcription effects depending on the HRE present (Glass et al., 1989). One DNA binding site of the TH receptor in the growth hormone gene obtained from rat pituitary GC_1 cells may contain additional regions 5' that provide a site for cooperative interaction with other transcription factors. (Glass et al., 1987; Hudson et al., 1990).

Role of protein-protein interactions. The receptor's ligand binding integrity is stabilized by association with core histones (Eberhardt et al., 1980; Ichikawa et al., 1987). However histone H1, which is associated with condensed, non-transcriptionally active chromatin (Littan, 1965), inhibits TH receptor binding to DNA (Ichikawa et al., 1987). Nuclear but non-histone proteins appear to enhance receptor binding to hormone response elements (Burnside et al., 1990). Therefore, other chromatin associated proteins can modify hormone binding to its nuclear receptor and receptor binding to DNA.

TH regulated gene products may themselves be transcriptional regulators. S-14, an hepatic gene believed involved in lipogenesis, is regulated by T_3, dietary, circadian, developmental and tissue specific factors. TH regulates transcription of S-14 via production of proteinaceous nuclear factors that function as transcriptional regulators and bind upstream from the S-14 start site (Wong et al., 1990). Since S-14 TREs occur far upstream from the cap site (Zilz et al., 1990), production of other cofactors may promote protein-protein interactions necessary to loop DNA and bring TREs closer to the promoter region (DeGroot et al., 1989).

The possible role of other co-factors. Recent evidence suggests that nonreceptor cofactors such as the early intermediate proteins fos and jun may enhance regulatory diversity and, depending on their ratios, either activate or

suppress TH dependent transcription (Diamond et al., 1990). These recently discovered transcriptional regulators are short-lived nucleoproteins, the synthesis of which is in response to extracellular signals (Nakajima et al., 1989; Sonnenberg et al., 1989) and dependent on the status of the cell (Seshadri and Campisi, 1990). T_3 stimulates growth in somatotrophic cells early in the G_1 phase (Defesi et al., 1985), the same phase in which fos and jun participate in proliferation of fibroblasts (Pardee, 1989). One of the early events following TSH stimulation of thyroid gland proliferation is the synthesis of fos (Colletta et al., 1986). Whether T_3 induces synthesis of fos and jun in the brain has not been explored.

Overview of thyroid hormone regulated transcription. The mechanism of hormone mediated transcription may be conceptualized simply as nuclear protein acting as a ligand dependent switch that evokes RNA polymerase activity. The evidence accumulated in recent years suggests that the control of transcription is enormously more complicated. Thyroid hormone receptor binds to chromatin immediately after synthesis but some steroid receptors are dependent on ligand-induced modification in the cytoplasm. Receptor binding to chromatin is nonrandom and requires not only specific DNA sequences but also may require other chromatin associated proteins such as histones. In addition, other nuclear cofactors such as early-intermediate proteins may enhance or suppress transcription. Phosphorylation of the receptor may modify transcription. "Dead" receptors that bind ligand but do not activate transcription would compete for ligand and dilute the available pool. Some erbA-like "receptors" may stimulate expression from TH response elements but without dependence on ligand binding as hypothesized for malic acid production in the brain (Strait et al., 1990).

Cooperative interactions between ligand occupied receptors, especially when multiple hormone response elements occur within the regulatory portions of the target gene, may impose yet another level of control that would depend on the level and organization of homo- or hetero-receptor saturation. In addition, TH binding may result in the synthesis of other transcriptional regulators as appears the case for T_3 regulation of S-14 gene transcription. The presence of a ligand-receptor-chromatin complex does not by itself predispose an all or nothing nuclear response. Like other transcriptional regulators, hormone binding may confer either positive or negative gene expression and regulate the magnitude of expression depending on cellular environment and tissue specific or developmentally specific factors.

Tissue ligand binding capacity and response in vivo. There is a correlation between receptor binding of T_3 or its analogs with in vivo responses (Eberhardt et al., 1980; Samuels, 1973). The determination of receptor concentration has been the focus of several investigations because the tissue concentration of receptors and their degree of saturation influences the magnitude of response (Oppenheimer 1983; Samuels et al., 1976; Samuels, 1983). Increased receptor occupancy down-regulates the number of receptors in cultured somatotrophic cells (Halperin et al., 1990; Raaka and Samuels, 1981). Halperin et al. emphasize that half-maximal in vivo responses such as cell growth and growth hormone synthesis occur at different levels of receptor saturation. Receptor density in fasted rat liver is approximately half that of non-fasted rats (Schussler and Orlando 1978). Depending on the system studied, increased TH may cause receptor concentration to decline (Samuels et al., 1976, 1977) as in pituitary GH_1 cells or the

neonatal rat brain (Schwartz and Oppenheimer, 1978), or increase (vonOverbeck and Lemarchand-Beraud, 1983). In constrast, T_3 is reported to augment ligand binding in developing pituitary nuclei (Coulombe et al., 1985). In hypothyroid neonatal rats, significantly higher T_3 binding capacity is observed in brain nuclei parallel to the low concentrations of hormone (Valcana and Timeras, 1978). Administration of T_3 to the hypothyroid rat causes an increase in the binding affinity of the liver nuclear receptor and a rapid reduction in maximal binding capacity (Ishiguro et al., 1980). Conversely, rat pancreatic nuclear receptors for TH appear to increase during the postnatal period and are up-regulated by thyroxine treatment (Lee et al., 1990).

In Xenopus larvae the accumulation of erbA transcripts preceeds competence to respond to hormone (Baker and Tata, 1990). The maximum accumulation occurs in thyroid responsive tissue and coincides with the serum thyroxine maximum during the metamorphic climax. These observations elicit the concept, albeit controversial, that TH may play a role in modulating the nuclear concentration of its receptor.

Determination of the maximal binding capacity may be misleading as multiple forms of the receptor exist and are differentially regulated by hormone concentrations (Hodin et al., 1990). Sodium butyrate differentially affects transcription of the TH receptor in nerve or glial-type cells (Yusta et al., 1990). Pituitary beta receptors are likewise affected by sodium butyrate; beta 2 receptor is repressed but no effect on beta 1 transcription is observed (Lazar, 1990). The T_3 binding capacity of rat brain increases 3-4 times during gestation (Perez-Castillo et al., 1985) and thereafter declines to adult values, 1.5 times fetal levels. There is a

sharp increase in the amounts of the beta 1 receptor mRNA during the period of postnatal brain development, increasing to about 40 times the fetal concentration by postnatal day 10 when adult levels are reached. The increase in brain beta 1 receptors does not appear causally related to T_3 content since the increase is also observed in hypothyroid rats. There does not appear to be a simple relationship between the alpha 1 and beta 1 mRNA content and the binding capacity suggesting a translational or posttranslational modulation of mRNA expression (Strait et al., 1990). This work suggests that the different members of the TH receptor family may have rather specific effects on transcription. For example, the beta 2 receptor is specific to the pituitary (Cook and Koenig, 1990; Hodin et al., 1990) and, therefore, may mediate TH participation in some endocrine functions. Secondly, T_3 enhances malic enzyme transcription in the liver but not in the brain which has substantial amounts of malic acid. The adult brain has high levels of the alpha 2 receptor which is not T_3 responsive (Cook and Koenig, 1990). It has been hypothesized that the preponderance of the non-T_3 responsive receptor in the adult brain uncouples the HRE from hormone control and allows the brain to produce malic acid for its metabolic needs (Strait et al., 1990). Lastly, the ratios of the different TH receptors is tissue specific and changes during the course of development (Strait et al., 1990)

FEEDBACK REGULATION OF THYROID HORMONE PRODUCTION

Thyroid histology (Nishioka et al., 1982) and the surge of serum thyroxine observed during smoltification in salmon indicates enhanced thyrotropic activity that is evidently not under feedback regulation. Surges of serum thyroxine also occur in the metamorphosing tadpole (Mondou and Kaltenbach, 1979) and the neonatal mammal. In the neonatal mammal, the hypothalamic-pituitary-thyroid axis may not be fully developed as briefly discussed below. However, smolting salmon and metamorphosing tadpoles are well developed, competent animals with presumably fully functional neuroendocrine homeostatic controls. Indeed, the thyroidal response to thyroid stimulating hormone is enhanced during smoltification (Specker and Schreck 1984).

In the typical vertebrate, TH represses the transcription of hypothalamic thyrotropic hormone (TRH) (Taylor et al., 1990), decreases the number of TRH pituitary receptors (Hinkle and Goh 1982), inhibits transcription of the beta subunit of pituitary TSH (Gurr et al., 1990; Wondisford et al., 1989; Wood et al., 1989) and thereby controls circulating levels of hormone. However, in rats TRH regulation of TSH is not apparent until several days after birth in spite of the presence of TRH mRNA in the parvocellular paraventricular hypothalamic nucleus (Taylor et al., 1990). Since the beta 2 TH receptor is specific to the pituitary (Cook and Koenig, 1990; Hodin, 1990), presumably mediating the pituitary responses of TH, ontogenic modulation of beta 2 levels or activities could uncouple TSH from TH feedback regulation.

Pituitary secretion of TSH in man varies daily and seasonally and apparently without TH feedback control as serum T_4 varies in parallel with TSH

(van Cauter and Ashchoff, 1989). In addition, TH affects the development of corticosterone diurnal rhythm (Lengvari et al., 1977). In this regard it is worth recalling that salmon smoltification seems to have a circadian component. Light pulses alter "pacemaker" activity and induce fos expression (Rusak et al., 1990) in the hypothalamic suprachiasmic nucleus which in turn entrains neuronal activity of other (slave) systems (Page, 1987). The altered nerve activity may affect the level of post-translational modifications such as phosphorylation which is known to affect ligand mediated transcription (Glineur et al., 1989). In addition, in vivo autoradiography suggests that both retina and pineal gland are major targets for TH during smoltification (Ebbesson et al., 1990). Melatonin is the major 5-hydroxyl tryptophan derivative produced by the pineal (Axelrod and Wurtman, 1968) and can modify the affects of TH (Wright et al., 1990).

The hypothalamic-pituitary-thyroid axis may not provide the only mechanisms regulating circulating concentrations of TH. Antibodies to the TSH surface receptor on thyroid cells mimic the activity of native TSH eliciting the synthesis of cAMP and, subsequently, T_4. Therefore, it has been suggested that some forms of Graves' disease are autoimmune diseases (Islam et al., 1983). While such chronic elevation of hormone concentrations may appear an inappropriate analogy to the episodic increases observed during smoltification, it does emphasize that agents other than TSH can stimulate the second messenger system that evokes hormone synthesis. Growth hormone may be thyrotropic in some animals (Grau and Stetson, 1979) and has been shown to stimulate peripheral deiodination of thyroxine to T_3 in salmonids (MacLatchy and Eales, 1990). Prostaglandins elicit the synthesis of cAMP and stimulate iodine uptake and

organification in thyroid cells in a manner similar to that of TSH (Takasu et al., 1982), but thus far the effects of prostaglandin treatment on circulating thyroxine in salmon has not been reported.

These suggested mechanisms and areas of future study emphasize that no consensus presently exists explaining the surge of circulating thyroxine which occurs during salmon smoltification and amphibian metamorphosis. Further, these observations suggest that the concept of feedback regulation may not adequately describe control of thyroid activity.

THE ROLE OF THYROID HORMONE DURING BRAIN DEVELOPMENT

Thyroid hormone serves as a general metabolic pacemaker in the adult animal (Hoch, 1974) but has profound, if poorly understood, effects on the developing organism. It is required for proper neural development (Ford and Cramer, 1977). For example, hypothyroidism during neonatal periods can cause severe mental retardation (cretinism) in man.

Nerve fiber proliferation, migration, and death. Thymidine autoradiography on hypothyroid rats clearly illustrates a prolonged phase of cell proliferation and a failure of cerebellar external granular cells to migrate through the molecular layer to the inner granular layer (Hamburg, et al., 1971). Such migration normally contributes to parallel fiber growth (Lauder, 1979). However, the effect of thyroxine on Purkinje cell maturation may be indirect (Hauser and Gona, 1984; LeGrand and Bout, 1971).

Quantitative Golgi methods have been used to demonstrate reduced dendritic fields in the caudate nucleus (Lu and Brown, 1977) and dendritic spine density in hippocampal pyramidal cells (Gould et al., 1990) that probably contribute to a persistently defective neuronal circuitry of hypothyroid rats. Collosally projecting neurons from the occipital and parietal cortices retain inappropriate immature projections to the contralateral cortex because of TH dependence on normal fiber elimination (Gravel and Hawkes, 1990; Gravel et al., 1990). It appears that reduced TH concentrations contribute to a failure of ontogenetic parcellation in this system. Neurite outgrowth and fiber proliferation followed by selective fiber elimination appears to be a principle feature of brain

development in the neonatal mammal, the metamorphosing frog, and smolting salmon. This "parcellation" (Ebbesson, 1980) of fiber input results in a neural re-organization dependent on the strength of the selective pressures encountered.

Mechanisms of nerve fiber proliferation and migration. Microtubule associated proteins (MAPs) affect the polymerization (Francon et al., 1977) and stabilization (Faivre et al., 1985; Matus, 1988) of tubulin and thereby affect axon growth (Matus, 1987; 1988) and the development of neural connections (Daniels 1972). MAPs are known to change during early brain development (Nunez, 1985; Riederer and Matus, 1985). Hypothyroid animals demonstrate a delayed development of adult forms of MAPs which can be compensated for by injections of thyroid hormone (Francon, 1977; Nunez, 1985). Further, MAPs from adult animals will initiate polymerization of tubulin from hypothyroid neonates. MAPs may be regulated by nerve growth factor (Greene et al., 1983) which in turn may be under some TH control (Aloe and Levi-Montalcini, 1980; Walker et al., 1981). Neurofiber maturation coincides with expression of neurofilament cytoskeleton proteins. Immunoreactivity to the 210 kd subunit of the neurofilament is suppressed in hypothyroid neonatal rats (Gravel and Hawkes, 1990).

The effects of TH on nerve growth factor is particularly interesting because there is evidence that a variety of nerve trophic factors are produced and some may be specific for particular classes of nerves (Hendry et al., 1983). Nerve growth factor is essential for the survival and, through interactions with TH, the enzymatic activity of some nerve cells (Hayashi and Patel, 1987). Inadequate trophic exchange or antibodies to nerve growth factor lead to neuronal death in specific types of neurons (Barde, 1989). Competition for trophic substances such as

nerve growth factor is a principal mechanism accounting for fiber and cell death (Perez-Polo et al., 1990). Insufficent trophic support by nerve growth factor underlies collateral elimination during normal development (Saffran and Crutchen, 1990). Interestingly, injection of nerve growth factor antibodies into the neonatal brain results in behavior "strikingly similar to that of hypothyroid rats" (Aloe et al., 1981). Insulin-like growth factor has been found in abundance in the olfactory bulb of salmon (Ebbesson et al., 1990) and massive nerve fiber proliferation occurs throughout the salmon brain during smoltification (Ebbesson et al., 1988 a,b). Many of the fibers acquired during smoltification do not survive into adulthood (Ebbesson et al., 1988b) suggesting that smoltification is similar to the mammalian neonatal state when large excesses of nerve fibers produced in the developing brain are not retained in the mature animal (Gravel and Hawkes, 1990; Gravel et al., 1990).

Not only does survival of nerve cells and fibers require trophic support from the synaptic target (Barde, 1989), but developmentally programmed cell death and fiber elimination requires gene activation (Schwartz et al., 1990). Neural activity, which enhances competition for trophic substances, determines the distribution of "synaptic weight" (Lisman, 1989) in the mature animal (Kandel, 1985). Neural activity induces fos expression (Doucet et al., 1990; Dragunow and Faull, 1989), which has been correlated with survival and maturation of granule cells (Didier et al., 1989).

TH affects the structure of the cell surface (Baxter et al., 1979; Garza et al., 1990) and influences the synthesis of hyaluronidase (Polansky and Toole, 1976). Hyaluronidase cleaves hyaluronate into smaller oligosaccharides (Toole,

1976). The concentration of the enyzyme changes in an orderly fashion in developing tissues (Polansky et al., 1974; Toole, 1973). In metamorphosing Xenopus, glycoproteins in the gut alter their lectin binding specificity (Ishizuya and Shimozawa, 1990) suggesting the onset of hyaluronidase activity. Because cell surface oligosaccharides are important for cell-cell recognition (Edelman, 1984), the regulation of hyaluronidase by T_3 may be one of the ways TH controls differentiation (Baxter et al., 1979). In this regard, cell surface recognition appears be an important component of nerve fiber migration (Edelman, 1984). To my knowledge, no determination of oligosaccharide products of hyaluronate breakdown in the brains of the neonate, metamorphosing tadpole, or smolting salmon has been reported.

Synaptogenesis (Salemi et al., 1990) and maturation of the synaptic plasma membrane, like cell migration and dendritic proliferation, are subject to a "critical period" dependence on TH (Lindholm, 1984; Rabié and Legrand, 1973). The reduced cell migration, dendritic and axonic growth, and synaptogenesis observed in the hypothyroid neonate may contribute to depauperate cortical electrical activity (Eayrs, 1960; Shapiro, 1968; Shaprio and Martin, 1976).

Other transmitters, receptors and effector units. TH modulates tissue sensitivity to other hormones and extracelluar signals. It regulates the number and activity of cell surface receptors and transduction from surface receptors by modulating expression of G-protein subunit mRNAs (Rapiejko, 1989) and regulates components of effector units such as adenylate cyclase (Rapiejko and Malbon 1987). Prompt action of T_3 on cellular metabolism may result from increased uptake of Ca^{+2} across the plasma membrane (Segal, 1990). Euthyroid

concentrations of TH are required for formation of the monoaminergic system in metamorphosing tadpole (Kikuyama, 1979) and for the synthesis and degradation pathways of 5 hydroxy tryptophan, norepinephrine, and dopamine in the postnatal rat (Singhal et al., 1975). Evidence that TH affects development of serotonergic (Savard et al., 1984) and cholinergic (Honegger and Lenoir, 1980) neurotransmitter systems is of interest because certain neurotransmitters exert developmental influences of their own (Lauder, 1983). The rate of change of neurotransmitter and amino acid concentrations in the brains of smolting salmon appears dependent on thyroid status (Ebbesson et al., 1989c).

Growth factors as mediators of thyroid hormone action. Growth hormone production is modulated by TH and cortisol (Martial, 1977). TH also affects the synthesis of epidermal growth factor and nerve growth factor (Walker et al., 1982; Walker et al., 1981). Growth hormone stimulated synthesis of insulin-like growth factor in the liver is amplified by T_3 (Fagin et al., 1989; Wolf et al., 1989). Binding capacity of the pituitary receptor for insulin-like growth factor is also increased by TH treatment of thyroidectomized rats (Matsuo et al., 1990).

The effects of TH on growth factors suggests a pleiotrophic effect on a variety of endocrine and autocrine factors (Fisher et al., 1982). Growth factors may be the principal intermediaries of TH action on the developing animal.

The tadpole analogy. Tadpole metamorphosis is conceptually very similar to salmon smoltification and has a long-recognized thyroid dependency (Allen, 1916). Thyroxine administration stimulates neural development in the metamorphosing tadpole (Kollros and Bovbjerg, 1990) and causes a two fold enhancement of hyaluronidase activity in the brain (Toole, 1976). Thyroid hormone

is preferentially localized along those portions of the neuraxis considered to be centers of neural adjustments necessary to support metamorphic transformation (Dratman et al., 1969).

Similar to nerve fiber proliferation during salmon smoltification, the development of new neural circuitry is a feature of tadpole metamorphosis. Hypothyroid Xenopus leavis larvae fail to develop a distinctive ipsilateral retino-thalamic projection which normally developes when thyroxine first becomes detectable in the tadpole. The projection from one eye can be rescued with an interocular injection of thyroxine at a dose which has no effect on the untreated eye (Hoskins, 1986).

Overview of thyroid hormone effects on brain development. Corticosteroids and TH appear to act in concert in coordinating spatio-temporal events in postnatal or metamorphic development (Lauder,1983). This interplay of hormones and transmitters on growth, development, and maturation emphasizes that brain development is an extraordinarily complex event. The heterogeneity of brain tissue, each cell group with its own schedule for maturation and susceptibility to TH induction (Oklund and Timeras, 1977) and the differential effects of hormone on different cell types (Hamburgh et al., 1971) precludes any simplified notion of brain maturation. However, the evolving consensus is that TH, either directly or via growth factors, affects nerve fiber proliferation, migration, and finally selective fiber elimination resulting in a neuronal organization capable of responding to selective pressure. The very first step in this cascade of events is the binding of the hormone to its receptor. Therefore, it is very pertinent that the number of TH receptors in the brain increases during fetal neuroblast proliferation

(Bernal and Pekonen, 1984) and postnatally coincident with a sensitive period of differentiation (Ferreiro et al., 1990; Perez-Castillo et al., 1985) but decreases overall with age (Schwartz and Oppenheimer, 1978). Based on the relative affinity for analogs (Ferreiro et al., 1987) and hybridization studies (Thompson et al., 1987), TH receptors in the developing brain may represent a different population than that of other tissues. This population of receptors regulates the transcription of a narrow portion of the genome and influences the most complex developmental event in an animal's life.

Neurological changes in salmon during smoltification. The onset of behavioral changes such as down-stream migration and olfactory imprinting imply that either preexisting neural circuits become activated by smolt transformation or that entirely new circuits are developed during this period. Recently it has become evident that, in addition to the gross morphological, osmoregulatory, and biochemical changes that characterize smoltification, the brain enters a period of neurological development.

Substantial changes in whole brain concentrations of dopamine, serotonin, gamma-aminobutyric acid and amino acids occur about the time serum thyroxine surges (Ebbesson et al., 1987; Ebbesson et al., 1989 a,c; Ebbesson et al., unpublished). Fish treated with propylthiouracil do not demonstrate either the same magnitude of change or the same rate of increase and subsequent decline in serotonin, dopamine, or norepinephrine (Ebbesson et al., 1989a). These changes are consistent with measurements of biogenic amines during altered thyroid states in mammals (Singhal et al., 1975)

Mitotic activity increases the volume of the olfactory bulb by 68% and is

accompanied by huge increases in the amount of insulin-like growth factor (Ebbesson et al., 1990). The olfactory bulb differentiates during smoltification with glomeruli becoming more clearly defined. Primary olfactory projections grow past the olfactory bulb to the ventral and ventro-lateral telencephalon (Bazer et al., 1987).

Retinal projections become unusually extensive as fibers invade the telencephalon, all layers of the optic tectum including the stratum griseum and periventricularis, the ipsilateral diencephalon, and the laminar nucleus of the torus semicircularis (Ebbesson et al., 1988 a,b). It is presently unknown whether the new fibers arise from new cell bodies or are collaterals. Most of the new fibers die off within a year after the fish enters salt water as the retinal projections of mature fish more closely resemble those of presmolt. The increased fiber growth, sometimes to unusual targets, is consistent with a period of increased growth factor induction of fiber proliferation (McGeer et al., 1987) and tubulin polymerization (Francon et al., 1977; Greene et al., 1983; Nunez, 1985). The influence of TH on growth factor production (Fagin et al., 1989; Walker et al., 1981; 1982) could indirectly affect the proliferation of nerve fibers (Saffran and Crutcher, 1990).

Substance P immunoreactivity is apparent and intensifies in the dorsomedial telencephalon, habenula, preoptic area, and the periventricular hypothalamus but declines in the pre-optic nucleus during smoltification (Ostholm and Ebbesson, 1989). Meanwhile, immunoreactivity to FMRF amide doubles in the retina and changes distribution in the optic tectum substantially (Ostholm et al., 1990b). FMRF amide immunoreactive cells become apparent in the nucleus of the terminal nerve, olfactory bulb and periventricular hypothalamus and invade part of

the olfactory mucosa (Ekstrom et al., 1988; Ostholm et al., 1989; Ostholm et al., 1990a). Olfactory FMRF amide-like immunoreactive cells appear to form connections with the retina and pineal (Ekstrom et al., 1988).

Circumventricular serotonin immunoreactive neurons such as pineal photoreceptors and periventricular hypothalamic cells increase in volume and number with age (Ekstrom and Ebbesson, 1989a). In areas of the brainstem (dorsal and medial raphe, dorsal lateral tegmemtum, left but not right habenula) serotonergic neurons increase in volume while serotonin immunoreactivity declines in portions of the reticular formation and the raphe pallidus (Ekstrom and Ebbesson, 1989 a,b). However, serotonin type 1 and type 2 receptors could not be found in smolting coho brains and muscarinic cholinergic receptors did not change nor were affected by propylthiouracil treatment during smoltification (Ebbesson and Kuhar, submitted). Both Substance P and serotonin are influenced by TH (Savard et al., 1984).

In vivo autoradiography indicates that large densities of specific T_3 receptors occur in the retina and pineal gland (Ebbesson et al., 1990). Substantial methionine incorporation has been observed in the pineal during smoltification (Ebbesson et al., 1990). The possible involvement of the pineal gland in smoltification has not been explored but may be related to a circadial rhythm in melatonin production (Axelrod and Wurtman, 1968). Melatonin affects TH activity (Wright, 1990). Ebbesson and Bazer have suggested that these neurological changes resemble a mid-life neonatal state (Ebbesson and Bazer, 1990).

Scholz's work indicates that behavioral changes and olfactory imprinting associated with salmon smoltification are dependent on TH (Hasler and Scholz,

1983). The known effects of TH on the synthesis of growth factors affecting fiber proliferation suggests that TH may control brain development by encouraging the tubulin polymerization, causing fiber elongation. Hyaluronidase mediated breakdown of complex oligosaccharides could assist cell recognition and adhesion allowing the fiber to locate its appropriate target during nerve fiber proliferation. Massive nerve fiber proliferation, which is now known to be a feature of salmon smoltification, is sculptured into the adult structure by fiber degeneration. The neurological substrate providing the basis for the changes in behavior and olfactory imprinting observed in smolting salmon is established sometime during the interval between proliferation and fiber degeneration.

The initiation of some of these events is likely preceded by hormone binding to specific members of the TH receptor family. It appears likely that a change in the relative or absolute concentrations of specific members of the TH receptor family may occur in the brains of smolting salmon similar to changes that have been observed in the neonatal rat.

Thyroid hormone binding studies on salmon. Parr induced to partially smolt with injections of TSH bind significantly more radiolabeled T_3 to brain cell nuclei and other thyroid sensitive tissues than do saline treated presmolts (Scholz et al., 1985). Secondly, Specker et al., (1984) noted that tissue capacity for TH increased during smoltification. These important observations suggest that binding affinity or maximal binding capacity of brain TH receptors increases in concert with increased thyrotropic activity.

Specific receptors for T_3 have been found in cell nuclei of thyroid responsive tissue in adult and post smolted salmon and trout (Darling et al., 1982; van der

and Eales, 1980; Darling et al., 1982). These receptors have dissociation constants similar to those observed for rat receptors (0.2-1.2 nM) and binding capacities that correlate with tissue response to hormone (Bres and Eales, 1990). Furthermore, binding capacity appears to depend on age and physiological status of the animal. The T_3 binding capacity of starved trout liver nuclei declines (van der Kraak and Eales, 1980), although not with the magnitude observed in starved rats (Schusser and Orlando, 1978). T_3 binding capacity of normal adult trout liver nuclei is 0.067 to 0.37 fM T_3/μg DNA) (Van der Kraak and Eales, 1980; Bres and Eales, 1986; Darling et al., 1982) depending on the age of the fish and experimental conditions. The receptor is a salt extractable protein associated with chromatin (van der Kraak and Eales, 1980; Lebel and Leloup, 1989). T_3 binding to the extracted protein demonstrates a similar K_D as the intact nuclei and a binding capacity of 0.16 fM T_3/ ug protein (Lebel and Leloup, 1989). These observations compare fairly well with those from rat pituitary (Larsen and Silva, 1983), cerebral hemispheres, and liver (Eberhardt et al., 1978) .

T_3 receptors in the adult salmonid pituitary appear to bind ligand with greater affinity than those obtained from gill or liver tissue. Further, the affinity of T_4 for the pituitary T_3 binding site is 3-5 times less than the affinity of T_4 for T_3 receptors from other tissues (Bres and Eales, 1990). Since pituitary receptors are believed to be exclusively beta 2 (Hodin, 1990; Cook and Koenig, 1990), this observation reinforces the notion that pituitary binding sites are not similar to T_3 sites elsewhere.

I am not aware of any published report on <u>in vitro</u> TH binding to brain nuclei from salmon undergoing the smolt transformation. Since 1) TH binding

capacity of the mammalian brain is suspected to change during sensitive periods of development, 2) a neonatal-like state of neurological change appears to occur during smoltification and 3) increased thyroid activity is required for olfactory imprinting, the binding capacity or affinity of TH to brain receptors may change during smoltification. The immediate goal of my research has been to measure the binding kinetics of the biologically active TH, T_3, to nuclear receptors located in the brains of smolting salmon and to determine if changes in the affinity or maximal binding capacity change during the course of smolt transformation.

HYPOTHESIS

Salmon smoltification is accompanied by changes in the binding of thyroid hormone to brain nuclear receptors.

MATERIALS AND METHODS

Materials. Hatchery reared coho salmon (Oncorhynchus kisutch) were generously provided by the Alaska Department of Fish and Game, Crooked Creek Hatchery, Soldotna, Alaska. ^{125}I 3, 5, 3' triiodothyronine 1200 uCi/ug (^{125}I T_3) was obtained from Dupont New England Nuclear. Unlabeled T_3 and all chemicals for buffers were obtained from the Sigma Chemical Company. Millipore UC polysulfone ultrafilters with 30,000 nominal molecular weight cutoff were used to separate bound from free ligand.

Methods.

Sample Collection. Coho salmon were collected from the hatchery at various periods during February to June, 1990. The collection period included presmolt, smolting, and down-stream migrants. Fish were anaesthetized in 3 aminobenzoic acid ethyl ester (MS222) and some individuals were weighed, measured and examined for external signs of smoltification (Cheek and Ebbesson, Addendum 1). The tails were severed, and blood from individual fish was collected from the caudal stump into 1.5 ml centrifuge tubes. The blood was allowed to clot on ice and centrifuged within 6 hours of collection at 10,000 g. The serum was pipeted to centrifuge tubes and frozen at -70°C until radioimmunoassay for serum thyroxine. The conus arterious was severed and the fish were bled to reduce cranial blood contamination of brain tissue. The cranium was opened and any residual pineal organ ablated with a positive displacement pipet. Each brain was removed to a solution of ice cold 0.32 M sucrose, 20% glycerol, 1.0 mM $CaCl_2$ (SGC). After collecting whole brains of 30 fish, the SGC solution containing the

samples was frozen on dry ice and stored at -70°C until homogenization.

Radioimmunoassay. Serum thyroxine (T_4) was determined by radioimmunoassay using a kit marketed by Abbott Laboratories (Tetrabead).

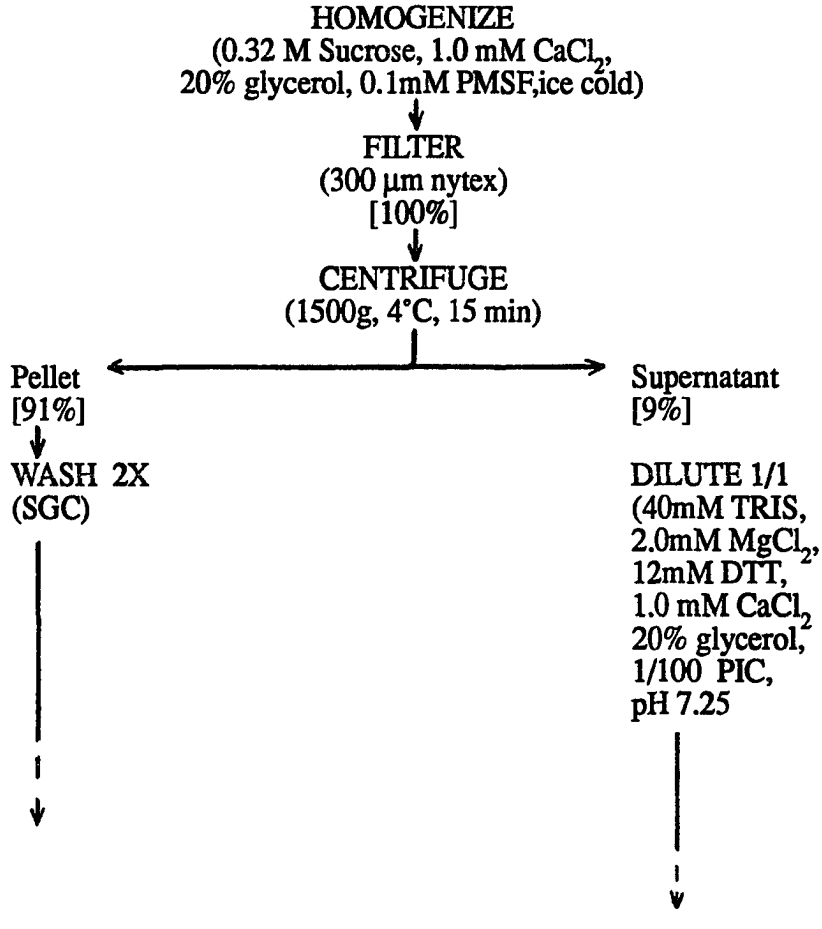
Tissue Homogenization. Each sample was thawed, drained, and homogenized in approximately 40 ml ice cold SGC with 0.1 nM phenymethylsulfonyl chloride in a glass Potter-Elvehjem homogenizer with a motor driven teflon pestle. The homogenate was filtered through 300 µm nytex and centrifuged at 1500 g and 4°C for 15 minutes. The 4°C temperature was maintained during all subsequent steps in the isolation protocol. The resulting supernatant was mixed 1/1 with pH 7.25, 40 mM TRIS buffer containing 1.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 12 mM dithiothreitol (DTT) and 20% glycerol and centrifuged at 10,000g and 4°C for 2 hours. The supernatant was frozen at -70°C for later examination of specific binding by soluble components.

The pellet resulting from the original 1500 g centrifugation was washed twice with SGC and resuspended in pH 7.25, 20 mM TRIS containing 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 6 mM DTT, 20% glycerol, 0.5% Triton X-100 and a 1/1000 final dilution of protease inhibitor cocktail (PIC, Kellogg et al., 1989) and allowed to sit on ice 5 minutes before centrifugation. The detergent was removed with two to three rinses of triton-free buffer and the pellet of cell nuclei resuspended in 10 ml pH 7.25, 20 mM TRIS containing 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 6.0 mM DTT, 20% glycerol and 1/1000 PIC (hereafter referred to as TMC), divided into 1.0 ml aliquots in 1.5 ml centrifuge tubes and frozen at -70°C until use. Samples from each stage of the purification protocol were analyzed for DNA by the method of Burton (1956). A flow chart of the isolation protocol is illustrated by Figure 3.

The percent recovery of homogenate DNA at each step of the isolation protocol (using tissues collected 22 May as an example) is shown in brackets.

Beginning with the tissues collected in mid-April, recovery of nuclear DNA from the homogenate started to decline significantly and the nuclear pellet was less labile to the perchloric acid digestion preceding analysis for DNA by the Burton method. Microscopic examination of the pellet following acid digestion indicated that most of the nuclei remained intact. It became necessary to disrupt the nuclear membrane with 0.4 M KCl prior to perchloric acid digestion in order to access the chromatin.

Flow Chart of Nuclear Isolation Protocol



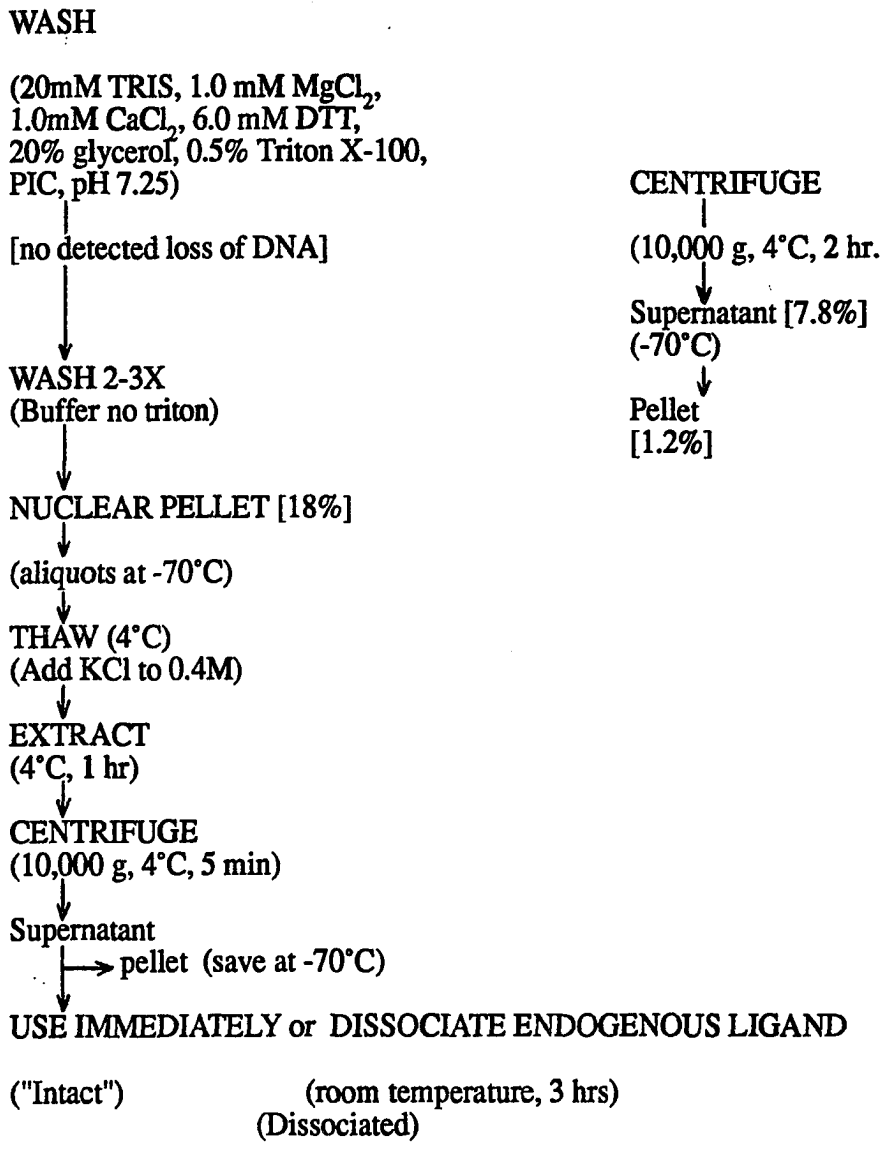


Figure 3. Flow chart of the nuclear isolation protocol. Percentages indicate measured recoveries of DNA at the designated step from the homogenization of tissues collected 22 May. Losses of nuclear DNA evidently occurred during the wash steps and when the pellet was moved into buffer, although no losses were observed when the pellet was washed with buffered detergent. Buffers are described in the text.

KCl extraction of nuclear proteins. Prior to exposing the receptor to ligand, 0.0298 g KCl was added to 1.0 ml of the nuclear suspension (0.4 M final

concentration) and allowed to extract at 4°C for 1 hour. This concentration of KCl is known to solubilize the nuclear receptor (DeGroot et al., 1974; Surks et al., 1975; Apriletti et al., 1988.). The uniform nuclear suspension degraded into an agglutinated mass that was centrifuged at 10,000g and 4°C for 5 minutes. The supernatant was pipeted off and either used immediately or frozen at -70°C. DNA and protein (Lowry et al., 1951) analyses of the supernate and pellet indicated that all the DNA was retained in the pellet.

Homologous displacement binding assay. Hormone binding was determined by an ultrafiltration assay. While either saturation binding or homologous displacement can be used to construct Scatchard plots, homologous displacement was the preferred method because an equal amount of information could be derived from fewer measurements. Millipore UC ultrafiltration tubes with a 30,000mw cutoff were loaded with pH 7.25, 20 mM TRIS, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 6.0 mM DTT, 20% glycerol and 0.4M KCl. All tubes contained identical concentrations of ^{125}I T_3 (usually 0.5nM). Six replicates containing only labeled T_3, six replicates containing labeled plus 100 molar excess unlabeled T_3, and 6 sets of 3 tubes each containing graduated amounts of unlabeled T_3 between 0 and 100 times the concentration of ^{125}I T_3 were used in each determination. The incubations were started by adding the extract obtained from 2 to 4 µg DNA to each tube. Final volume was 350 µl. The tubes were incubated at 4°C for 20-24 hours. 4°C was chosen as the incubation temperature because it was physiologically relevant and because temperatures lower than 20°C slow degradation of the receptor (Surks et al., 1973). The incubation was terminated by centrifugation at 16,000g 4°C, 15 minutes. The filters were washed 3 times with TMC plus 0.4 M KCl and

0.5% Triton X-100 and counted.

If the filters were washed with only TMC plus 0.4M KCl, up to 80% of the radioactivity was retained non-specifically by the filter. It was determined that 3 washes with 2% triton in buffer reduced artifactual binding to 1-2%. However, 0.5% triton in buffer reduced artifactual binding to 6% with a slight decrease in variability. Blanks were run with each experiment to confirm the level of artifactual non-specific binding.

The "Ligand" program (Munson, 1987; Munson and Robard, 1980) was used to analyze the data. The program determined non-specific binding from the limit of bound/free ligand (Chamness and McGuire, 1975) and subtracted non-specific from total binding to determine specific binding. Several such determinations were made on each tissue preparation and the data merged on the "Ligand" program. The dissociation constant (K_D) was obtained directly and the binding capacity (n) in fM T_3/ μg extracted DNA calculated from the results of the least squares minimalization of the binding parameters by the program. Differences in binding parameters between the various sampling periods were determined by the student's t test.

Maximal Binding Capacity. Because the local T_3 concentration is unknown and not likely directly related to serum T_4 concentration because of variable rates of peripheral tissue deiodination (Fok and Eales, 1984), the saturation of receptors by endogeneous hormone cannot be determined directly. Instead, percent occupancy was determined indirectly from parallel experiments on "dissociated" receptors. Receptors were dissociated by incubation at room temperature for 3 hours. The increased temperature promoted dissociation of

endogenously bound hormone from the receptor (Lee et al., 1990), but also may promote some degradation of the receptor (Van der Kraak and Eales 1980; Ichikawa et al., 1989). The total binding capacity determined on dissociated receptor was defined as the maximal binding capacity. The ratio of the difference in binding capacity measured in non-dissociated and dissociated nuclear extract to the maximal binding capacity was defined as the percentage of maximal binding capacity occupied by endogenous hormone.

Dissociation Rate. The rate at which the ligand-receptor complex decomposed to unoccupied receptor and free ligand was determined on dissociated receptor preparation that had been labeled with $^{125}I\ T_3$ as above and collected on Millipore ultrafilters. In some replicate experiments, receptor was labeled in a different concentration of ligand to determine if the dissociation rate changed dependent on the degree of saturation. The unbound ligand was removed by centrifugation and the filters washed 3 times with TMC, 0.4M KCl with 0.5% Triton X-100, and once with detergent-free buffer. The labeled receptor preparation was resuspended in TMC, 0.4M KCl buffer containing 50 nM unlabeled T_3 and the tubes incubated at 4°C for variable periods up to 32 hours. The second incubation was terminated by centrifugation and three detergent buffer washes as above. The retained radioactivity was used to calculate the amount of bound $^{125}I\ T_3$. The data were corrected for background and for the differences in binding anticipated from using different concentrations of $^{125}I\ T_3$ to label the receptor. The results were subjected to regression analysis where

$$B^* = -v_r t + B_o \qquad (1)$$

(B^* = labeled ligand concentration in fM $T_3/\mu g$ DNA, v_r= dissociation rate, t=

time, and B_o = maximal binding at t = 0). The dissociation rate constant, k_{-1}, was calculated from the relation

$$v_r = k_{-1}(nP_tA) \quad (2),$$

where nP_tA is the maximal binding capacity. Given the receptor saturation and the duration of the experiment, the dissociation rate was assumed to be linear. Significant differences between the slopes, and thereby the dissociation velocity, were determined with the student's t test.

The student's t test was confirmed by regressing pairs of data together and testing for goodness of fit with an extra sum of squares F test. However, in order to compare the slope of the dissociation curve from different tissue samples, it was necessary to establish a y intercept of constant value. The intercept determined from the data pooled from three dissociation experiments on tissues obtained 6 June was used to normalize the data acquired from each previous determination. The normalized data from each determination was subjected to regression analysis along with those from 6 June and examined for goodness of fit by the extra sum of squares F test (Munson and Robard, 1980).

The dissociation rate constant k_{-1} was calculated from rate of dissociation ($v_r = k_{-1}[nP_tA]$ where nP_tA = maximal binding capacity). The association rate constant (k_{+1}) was calculated from the relation

$$K_D = k_{-1}/k_{+1} \quad (3).$$

Equations

The binding equation for A ligand bound to n sites on P receptors is

$$nP + A \underset{k_{+1}}{\overset{k_{+1}}{\rightleftharpoons}} nPA \qquad (4)$$

$$\frac{k_{+1}}{k_{-1}} = K_A \qquad (5)$$

$$K_A = \frac{[nPA]}{[nP][A]} \qquad (6)$$

$$\text{with units } L/M = \frac{(M/L)}{(M/L)(M/L)}$$

The binding constant, K_A, is valid assuming that all binding sites have equal, fixed affinities that do not vary with the extent of occupancy.

The inverse of Eq. 6 is

$$\frac{k_{-1}}{k_{+1}} = K_D, \qquad (3)$$

the dissociation constant.

The forward rate is

$$v_F = k_{+1}[nP][A] \qquad (7)$$

$$\text{with units} \quad M/t = \frac{1}{M \cdot t}\frac{(M)(M).}{(M)}$$

The reverse (dissociation) rate

$$v_r = (k_{-1})[nPA] \quad (8)$$

with units M/t, where t = time, describes first order kinetics and appears linear so long as the number of binding sites occupied doe not become small. The amount of A bound is

$$B = [nPA] = K_A [nP][A] \quad (9)$$

$$\text{and } nP_t = nP + B \quad (10)$$

is the total amount of receptor. How many of total available receptor sites are occupied at a given [A]?

Dividing the amount bound by the total receptor

$$\frac{B}{nP_t} = \frac{K_A[nP][A]}{[nP] + K_A[nP][A]} \quad (11)$$

and dividing ou $\frac{B}{nP_t} = \frac{K_A[nP][A]}{[nP] + K_A[nP][A]}$

$$\frac{B}{nP_t} = \frac{K_A[A]}{1 + K_A[A]}. \quad (12)$$

Since $K_A = 1/K_D$,

$$\frac{B}{nP_t} = \frac{[A]}{K_D + [A]}, \quad (13)$$

the Michaelis-Menton equation.

From this relation it is possible to determine the total number of receptor sites and the dissociation constant from the amount bound over a range of [A]. Inversely, if K_D and nP_t are known, [A] can be estimated. However, it is usually convenient for mnemonic purposes to express the equation in linear form. We wish to express K_D and nP_t in a form that is readily conceptualized graphically.

Divide numerator and denominator of the right side of Eq. 13 by [A]

$$\frac{B}{nP_t} = \frac{\frac{K_A[A]}{[A]}}{\frac{1 + K_A[A]}{[A]}} \qquad (14)$$

cross multiply and rearrange

$$B = -K_A \frac{B}{[A]} + K_A nP_t \qquad (15)$$

This is the Eadie/ Scatchard equation. It states that the ratio of bound ligand to free ligand is linearly related to the amount of bound ligand with a negative slope equal to the binding affinity. Where [A] becomes very large and B/A approaches zero, **B** approaches nP_t or the binding capacity.

Terms

Symbol	Definition	Units
B*	Labeled ligand bound at t	$\frac{fM\ T_3}{\mu g\ DNA}$
B_O	Labled ligand bound at t = 0 Normalized to the concentration of sites occupied at the concentration of ligand used to label the receptor.	$\frac{fM\ T_3}{\mu g\ DNA}$
K_A	Intrinsic binding constant or binding affinity. The binding constant is the inverse of the dissociation constant.	L/M

K_D	Dissociation constant. Equals the concentration of ligand required to saturate 1/2 of the available sites and is a convenient way of understanding binding affinity because it is in the same units as [A].	M/L
k_{-1}	Dissociation rate constant. A constant fraction of the ligand-receptor complex decomposes to free ligand per unit time.	1/t
k_{+1}	Association or binding rate constant. Free ligand binds to unoccupied receptor sites at a constant rate over time.	1/M·t
n	number of binding sites per unit of receptor	
nP_tA	Binding capacity. The concentration of ligand that can bind to the material extracted from 1.0 µg DNA. Some receptors in the salt extract were occupied by endogenous T_3 that was present when the fish was sacrificed.	$\frac{fM\ T_3}{\mu g\ DNA}$
nP_tA (MBC)	Maximal binding capacity. The concentration of ligand that can bind to the salt extract obtained from 1.0 µg DNA. The salt extract had been incubated at room temperature for 3 hours to dissociate endogenously bound T_3; therefore, all possible binding sites were available to ligand at the beginning of incubation.	$\frac{fM\ T_3}{\mu g\ DNA}$
t	time	hours

v_r Dissociation rate.
Ligand binding is reversible. When the number of sites occupied is large, the rate of dissociation approaches linearity over time, i.e., appears like zero order kinetics, with a constant fraction of occupied sites dissociating per unit time (dissociation rate constant, or k_{-1}). However if 100% dissociation were allowed, ligand liberated per unit time slows and the slope of rate versus time becomes curved, i.e., second order kinetics.
1/t

Definitions and abbreviations

"Intact" receptor preparation
Salt extract of nuclear DNA that had been maintained at 4°C throughout the procedure. Some receptor sites in this material were occupied by T_3 that was present when the fish were sacrificed.

"Dissociated" receptor preparation

Salt extract of the nuclear DNA that had been warmed to room temperature for 3 hours before incubating with ^{125}I T_3. The warmer temperature promoted dissociation of endogenously bound T_3.

DTT dithiothreitol

Maximal Probable Percent Occupancy

Maximal probable percent occupancy is derived from the 95% confidence intervals of maximum binding capacity and binding capacity. The difference between maximum binding capacity and binding capacity is the concentration of sites occupied by endogenous T_3. The ratio of endogenously bound sites to the maximum binding capacity is an estimate of the degree of receptor saturation at the time the fish were sacrificed.

PIC	Protease inhibitor cocktail consisting of 1.0 mM benzamidine HCl, 0.1 mg/ml phenanthroline, and 1.0 mg/ml each of aprotinin, leupeptin and pepstatin A in ETOH was stored at -20°C and warmed in the hand and stirred before dispersal.
RIA	Radioimmunoassay
SGC	0.32 M Sucrose, 20% glycerol, 1.0 mM $CaCl_2$
TMC	20 mM TRIS, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 6.0 mM dithiothreitol, 20% glycerol, 1/1000 PIC, pH 7.25
TH	Thyroid hormone, T_3 or T_4.
T_3	3,5,3' triiodothyronine, is the principal biologically active iodothyronine.
nT_4	3,5,3',5' tetraiodothyronine, the principal circulating iodothyronine and is the "prohormone" and precursor of T_3.

RESULTS

Serum thyroxine. Figure 4 illustrates the changes in serum thyroxine concentrations measured from blood obtained from coho salmon before and during the smoltification metamorphosis. The broad arrow at 3 June represents the day fish were released from the hatchery. Hatchery personnel determined release date, in part, by the extremely agitated behavior of the fish and their tendency to crowd the lower part of the raceway, indicative of migratory predispostion. This year the behavior was initiated by a sudden increase in water temperature to 12°C which, combined with local crowding, reduced dissolved oxygen. Hatchery workers kindly retained a limited number of these down-stream migrants to enable tissue collection on the 6 June sampling period.

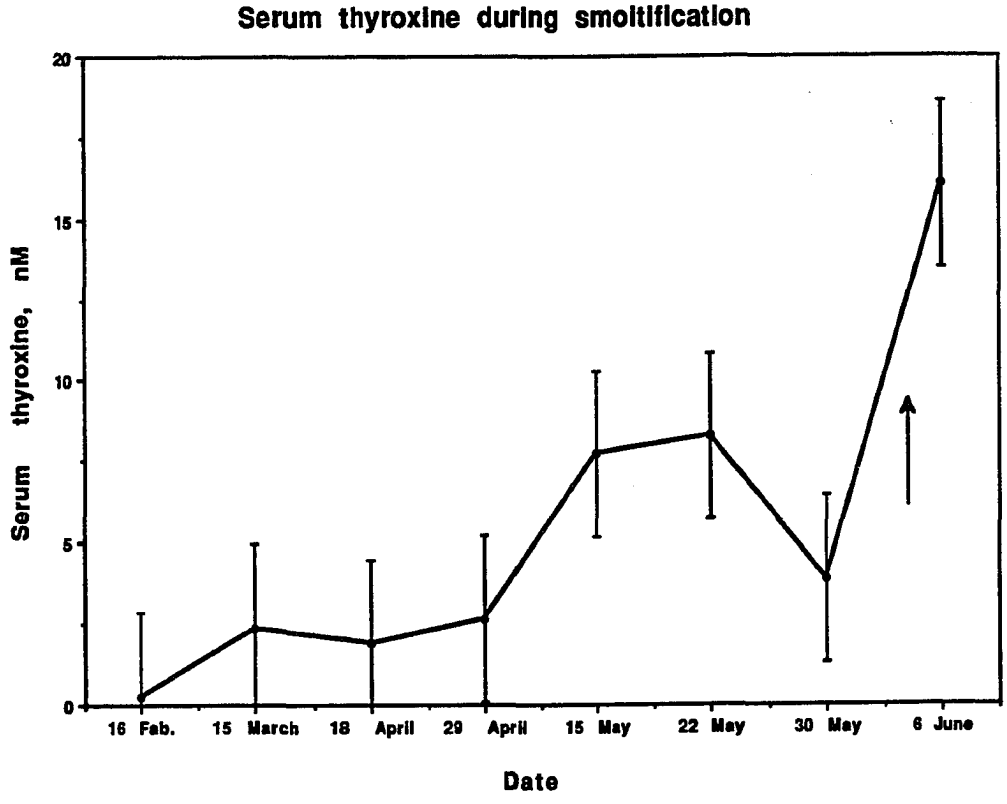


Figure 4. Serum thyroxine concentrations measured on coho salmon from Crooked Creek hatchery fish during 1990. Error bars are 95% confidence intervals. The broad arrow indicates the date intense migratory behavior caused hatchery personnel to release the stock. Fish sampled after this date were retained by the hatchery for this experiment and are considered migrants. RIA by the Abbott Tetrabead procedure.

Smoltification stage. Figure 5 illustrates the stage of smoltification based on a series of subjective determinations of pigmentation (loss of parr marks, silvering, darkened tips of the dorsal and tail fin), dermal thickening on the snout, and the appearance of palpebrae as described in Addendum 1. The bar graph suggests that the changes in morphology and coloration normally associated with

smoltification began to occur during mid May and are completed prior to down-stream migration. However, 1990 coho at the Crooked Creek Hatchery did not survive in salt water when challenged on 30 May (hatchery records), 4 days prior to their necessitated release.

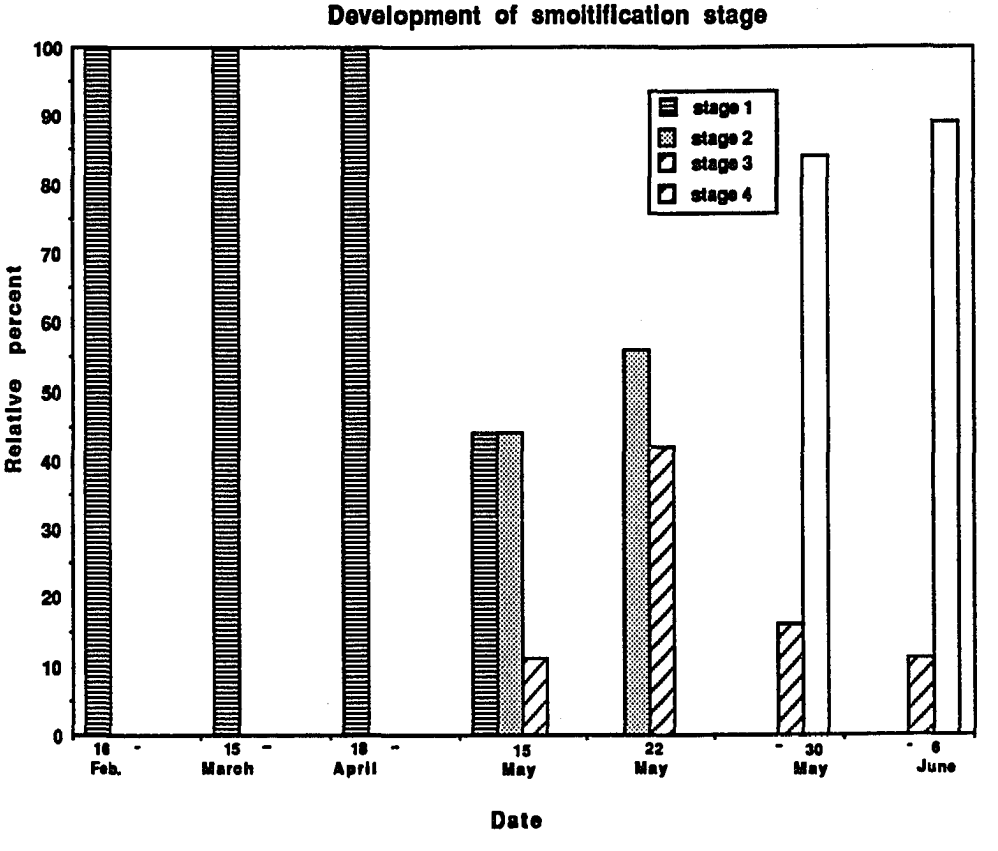


Figure 5. Development of the external indices of smoltification during the course of the sampling period. The characteristics of each stage are described in Addendum 1.

Yields of nuclear DNA. The recoveries of nuclear DNA from the tissue homogenate of each sampling period are presented in Figure 6. The decline in nuclear DNA recovery, from 70 to approximately 30% percent yield beginning with samples collected during mid-April, suggests that the extraction protocol

enhances selection of a distinct subpopulation of nuclei (Bellard et al., 1989). Indeed, in prior years efforts to isolate intact nuclei failed when homogenization was done in 0.32M sucrose: beginning about mid-April, nuclei would disintegrate when the suspension was moved into buffered triton. This problem was at least partially resolved by the addition of 20% glycerol (Sheer et al., 1985) and 1 mM $CaCl_2$ to the homogenization medium in an attempt to stablize the nuclear membrane. Also beginning about mid-April, the resulting nuclei were not amenable to perchloric acid oxidation using the published digestion protocol. This required disruption of the nuclear membrane with 0.4M KCl before DNA could be analyzed by the Burton method.

While microscopic examination of the final nuclear suspension indicated low amounts of debris and nuclear aggregation, the protein/DNA ratios exceeded 2 indicating that soluble protein contaminated the final nuclear pellet. An additional TMC wash appeared necessary to reduce protein/DNA to 2-3. As discussed below, the contaminating protein did not appear to have binding activity, as specific binding could only be observed when the nuclear proteins were solubilized in 0.4M KCl.

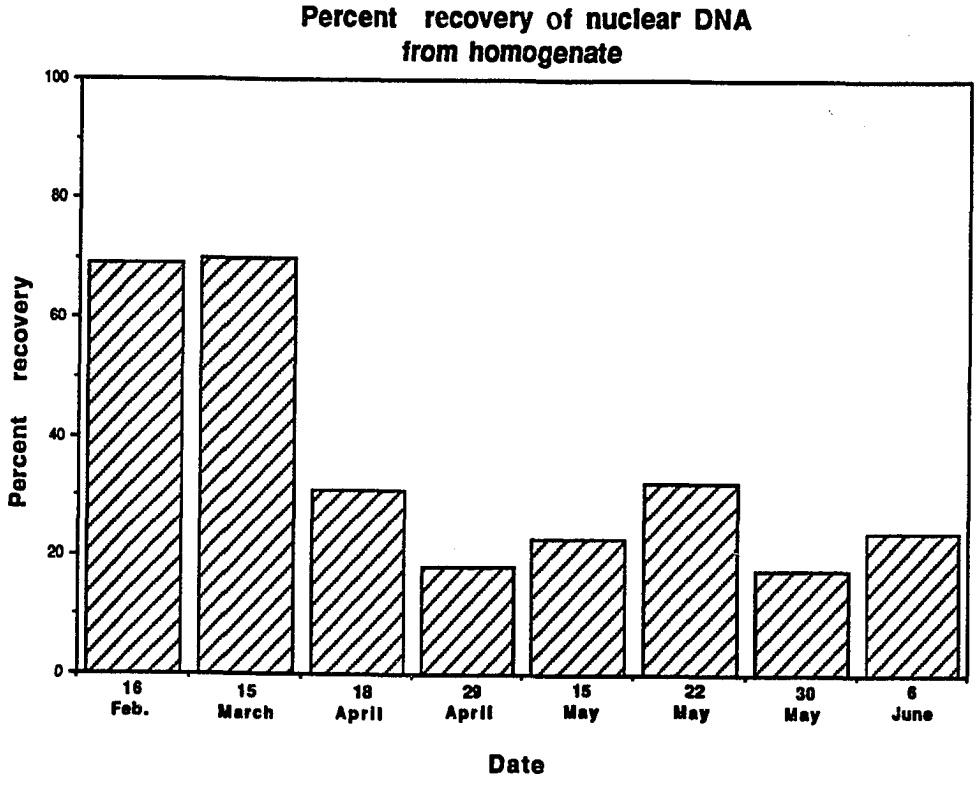


Figure 6. Recovery of nuclear DNA from the tissue homogenate. All samples were homogenized in SGC and assayed for DNA by the Burton method as described under Methods.

Requirement for KCl extraction. Figure 7 illustrates the solubilization of receptor when intact nuclei were incubated with $^{125}I\ T_3$ in 1.5 ml centrifuge tubes with various concentrations of KCl added to the medium. After centrifuging, the pellet was counted and the supernate containing extract was subjected to ultrafiltration as discussed under Methods. The figure shows the amount of radioactivity retained by the ultrafilter and pellet. A slight, but not significant, increase in binding by the pellet was observed using 0.2 M KCl in the medium. The 0.2 M

KCl was sufficent to degrade the nuclear suspension to an amorphous mat but was probably insufficent to extract receptor from the DNA (DeGroot et al., 1974; Torresani and DeGroot, 1975). It is apparent that a minimum of ligand binding occurred when the isotope was incubated with intact nuclei under low salt conditions. Further, very little bound radioactivity was recovered on ultrafilters at lower salt concentrations indicating that neither soluble protein contaminants nor "leaked" receptor (Ichikawa and DeGroot, 1987) accounted for significant amounts of binding. Filter bound radioactivity increased significantly when nuclear proteins were solubilized in buffered 0.4M KCl. I was consistently unable to reproducibly demonstrate specific binding of T_3 to intact nuclei from any preparation but could readily do so on the salt extract. This result convinced me to abandon attempts to demonstrate specific binding to intact nuclei and to conduct binding experiments on the salt extract.

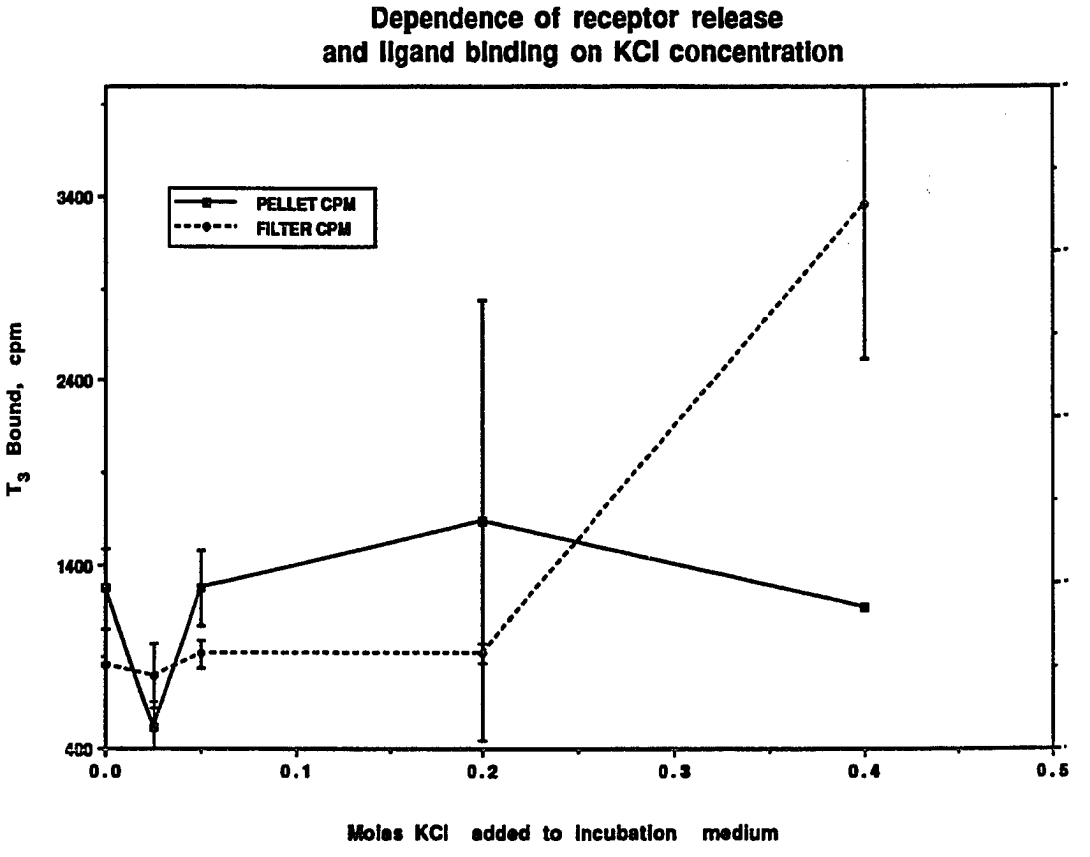


Figure 7. Radioactivity bound in nuclei or solubilized and retained by 30,000 mw. ultrafilters when nuclei were incubated with various concentrations of KCl. Error bars are standard deviations.

Receptor concentration dependence. Figure 8a demonstrates that the amount of radioactivity retained by the 30,000 mw. cut-off ultrafilters depends on the amount of KCl nuclear extract added to the incubation medium. The data indicate that total binding is dependent on the amount of extract incubated with the labeled ligand. At higher ligand concentrations the amount of bound radioactivity tends to decline, but the differences are not signficant.

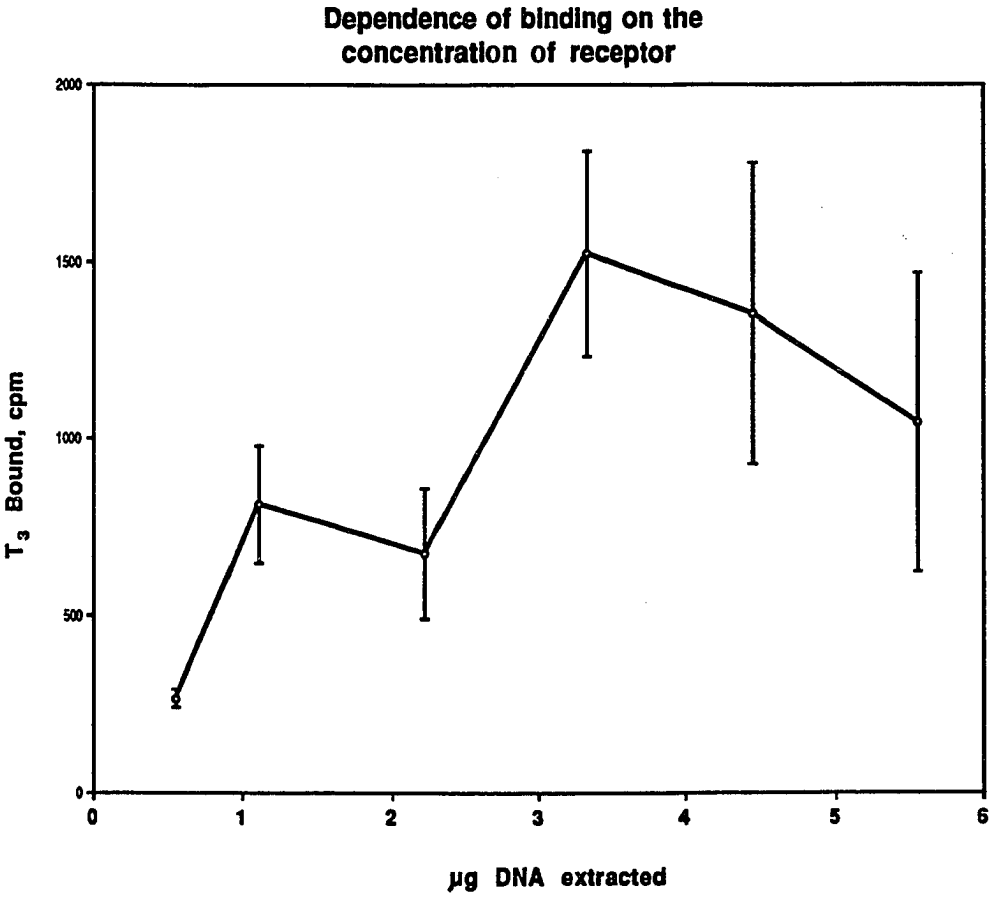


Figure 8a. Radioactivity retained by 30,000 mw ultrafilters when various concentrations of nuclear extract were incubated with ^{125}I triiodothyronine. Error bars are standard deviations.

Equilibrium assumption. Several preliminary experiments led to determination of the time required to reach equilibrium. These were conducted under slightly different conditions of temperature, salt load, and concentration and cannot be directly compared to the result illustrated in Figure 8b and consequently are not shown. Nonetheless, they did suggest that equilibrium required greater than 4 hours when the receptor was incubated at 4°C. The figure indicates that a maximum of radioactivity is retained by the ultrafilters after a 24 hour incubation.

The result was considered sufficent to satisfy the equilbrium assumption and establish conditions for displacement binding assays. A requirement for prolonged incubation periods to reach equilibrium at lower temperatures has been observed previously (Bernal et al., 1978; Galton, 1988; Lee et al., 1990). Incubation extended over a period of days appears to lead to a rather slow but significant receptor degradation which is occasionally observed in binding assays (D. Baskin, pers. comm.).

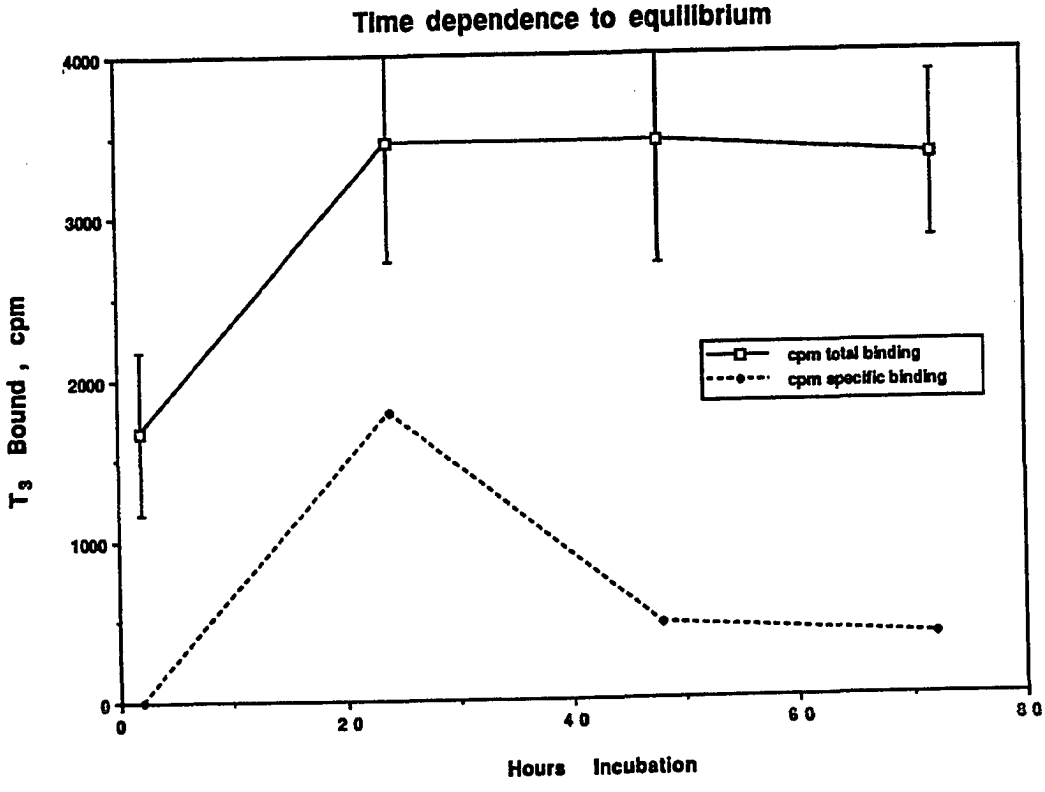


Figure 8b. Total binding and specific binding trapped on ultrafilters as a function of the duration of the incubation. Error bars are standard deviations.

Typical homologous displacement results. Figure 9 is a plot of

homologous displacement binding of $^{125}I\ T_3$ with unlabeled ligand on receptor preparations obtained from 16 Feb. specimens. Several experiments using 0.1 to 0.75 nM labeled T_3 were merged on the "Ligand" program to produce the plot. The observed data are represented by open circles, while the values determined by the least squares minimalization of the parameter values by the "Ligand" program, are represented as closed circles. The curve illustrates the expected sigmoidal shape and confirms that receptor saturation is achieved under the conditions of the assay (Boeynaems and Dumont, 1975; Klotz, 1982).

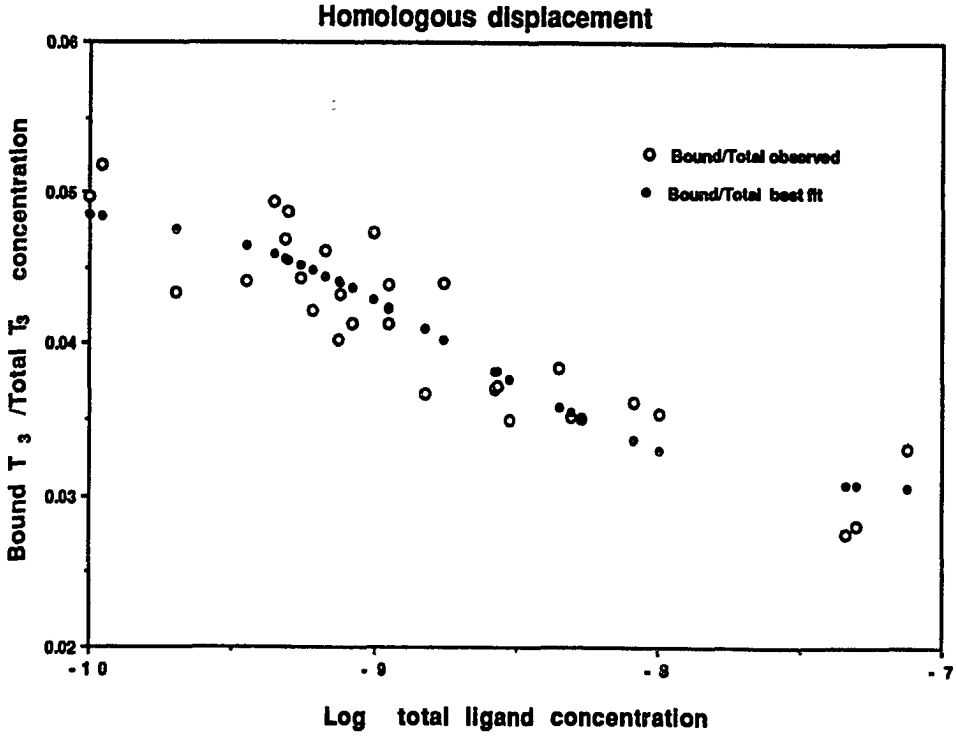


Figure 9. Homologous displacement of labeled T_3 by unlabeled T_3 on KCl extracts of brain nuclei obtained 15 Feb. Three separate experiments of 0.1,0.5 and 0.75 nM $^{125}I\ T_3$ were used to label the receptor. Observed values are designated as open circles and the least squares fitted values are shown as filled circles.

Figure 10a is a Scatchard plot of the same data presented in Figure 9. The data have not been corrected for nonspecific binding. The fitted values are again rendered as dark circles. The figure illustrates that receptor binding reaches an asymptote, the limit of B/F. The limit B/F is an estimate of the level of binding expected at very high ligand concentrations, i.e. unsaturable or non-specific binding. Non-specific binding is usually low affinity, high capacity binding that is not readily saturable. It includes binding to the surfaces of the apparatus (artifactual binding) and to low affinity sites in the receptor mixture. The unsaturability of the preparation is illustrated by Figure 11 which is a plot of binding versus ligand concentration. Total binding, which includes specific and non-specific binding, is represented by open circles. It can be seen that total binding is linear with ligand concentration and shows no indication of saturation within the concentration limits illustrated. The data corrected for non-specific binding are rendered in black and illustrate that specific binding approaches saturation. Subtraction of the limit B/F (i.e. where the slope of B/F versus B approaches zero) from total binding is an accurate method of obtaining specific binding (Chamness and McGuire, 1975). A Scatchard plot of the data corrected for non-specific binding is illustrated in Figure 10b where the fitted values are shown in black.

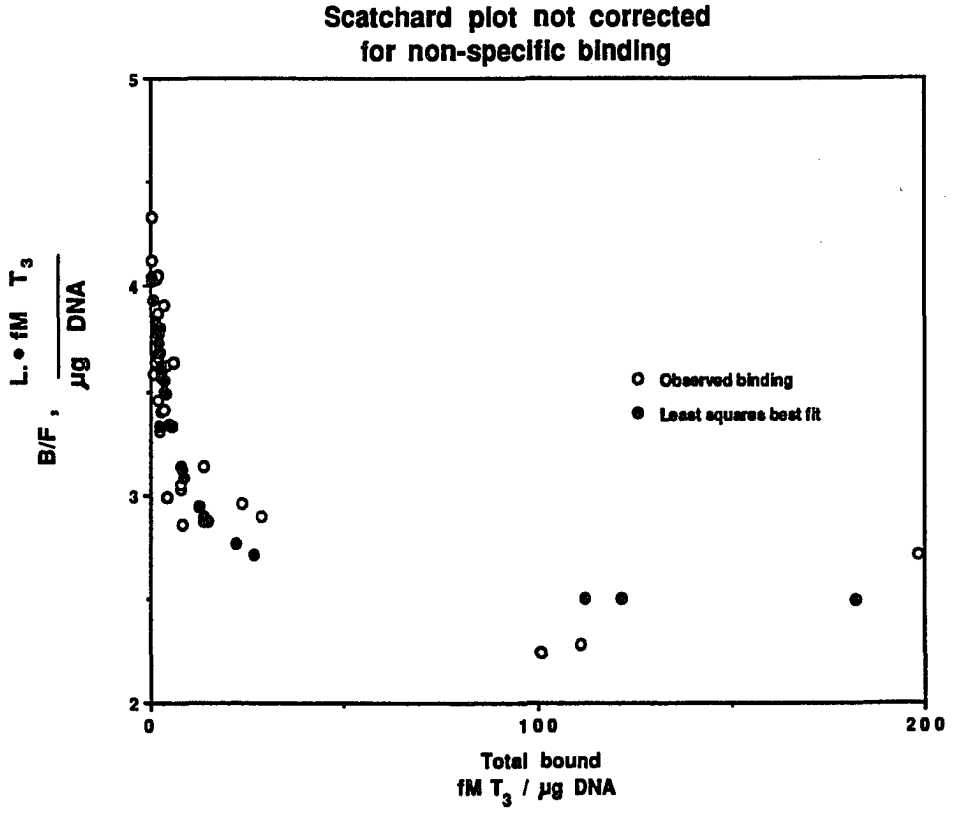


Figure 10a. Scatchard plot of the data from Figure 9 not corrected for nonspecific binding. The open circles are the observed values for total binding whereas the filled circles are the least squares fitted value. The ratio of B/F reaches an symptote, the lim B/F, or the level of nonspecific binding in this experiment.

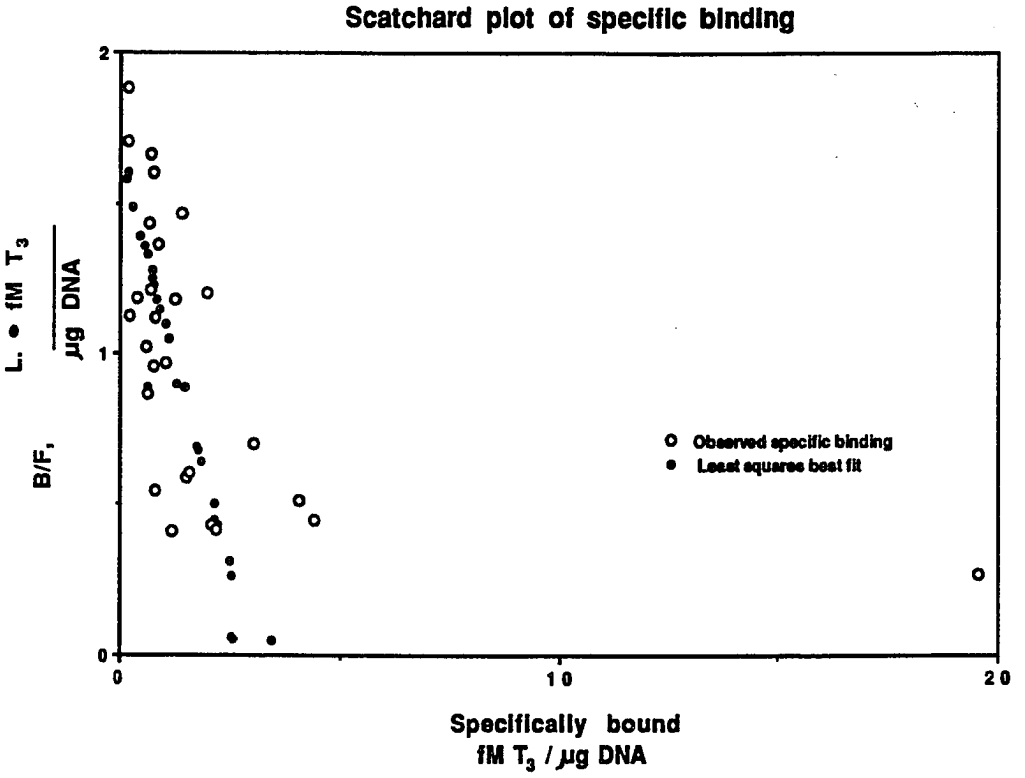


Figure 10b. Scatchard plot of the data from Figure 9 corrected for nonspecific binding. The open circles are the observed values whereas the filled circles are the least squares fitted values after correcting total binding for nonspecific binding. Nonspecific binding is the asymptote, lim B/F, illustrated in Figure 10a.

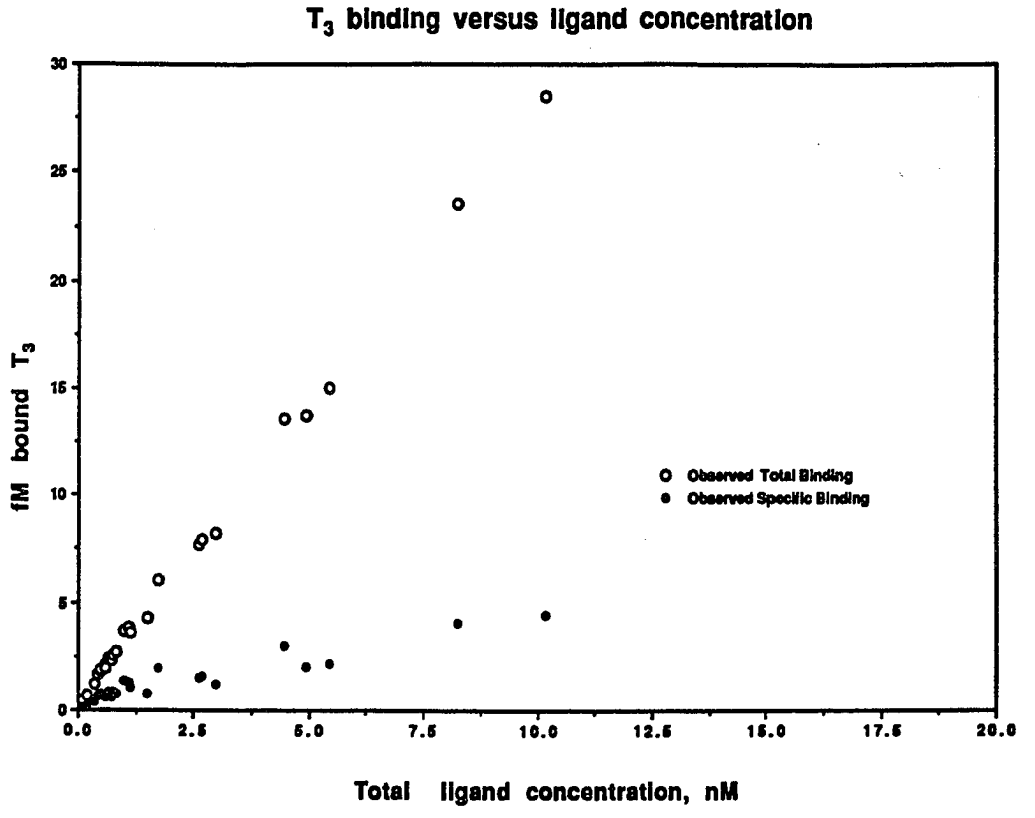


Figure 11. A plot of total binding and specific binding of the data from Figure 9 versus the total concentration of labeled and unlabeled ligand. Total binding does not indicate saturation indicative of low affinity, high capacity sites present in the measurement. The highest three concentrations used were deleted from this figure for scaling purposes.

The "Ligand" program calculates the predicted values based upon the non-linear least squares minimalization of the relation bound/total versus the log of the total ligand concentration and determines the limit of bound/total ligand as an estimate of the level of non-specific binding. These values are accessible through the program and were used to calculate points suitable for Figures 9, 10a, 10b and 11. In all cases the level of nonspecific binding is appreciable and largely

attributable to artifactual binding by the apparatus.

Sources of error. Some of the ultrafilters demonstrated retention of radioactivity that was extremely inconsistent within a given concentration replicate. These presumed outliers were deleted from the initial non-linear regression and reinserted, one at a time, until the "Ligand" program was unable to calculate a curve without substantial error. Since most homologous displacement experiments were repeated a minimum of three times and the data merged on "Ligand", the effects of such variability within replicates of a given experimental series could be reduced (Munson and Robard, 1980). However, the variable isotope retention by individual filters cannot be eliminated and accounts for substantial error in the determination of the binding parameters.

Systematic errors may be introduced. Each tissue sample is a mixture of brains from fish at different stages of smolt transformation (Figure 5). Those samples where no one stage seems to dominate the mixture could express constitutive errors.

Results of homologous displacement and dissociation rate experiments. The values for the dissociation constant (K_D) and the binding capacity for the intact and dissociated receptor preparations obtained from salmon brain throughout smoltification are given in Table 1. Missing from these data are the results from late March. Nuclear extracts from this time period failed to demonstrate measurable specific binding.

Table 1

Results of T_3 equilibrium binding and kinetic experiments conducted on salmon brain nuclear extracts throughout smoltification

Date	K_D, nM (95% CI)[A]	nP_t, fM T_3 / µg DNA (95% CI)[A]	Dissociation Rate, fM T_3 / µg DNA·hr (95% CI*)[B]
16 Feb(intact)	1.81 (1.01-3.26) n=23	3 (1.38-6.53)	
6 Feb (dissoc.)	1.29 (0.77-2.16) n=16	1.23 (0.86-1.76)	0.513 (0.648) n=14
18 March(intact)	0.417 (0.398-0.437) n=28	1.49 (1.11-1.97)	
18 March(dissoc.)	0.861 (0.53-1.4) n=10	1.54 (0.85-2.8)	0.158 (0.083) n=22
29 April(intact)	0.51 (0.472-0.551) n=12	3.0 (1.38-6.51)	
29 April(dissoc.)	1.71 (0.52-5.63) n=4	1.9 (0.65-5.55)	0.0884 (0.154) n=17
15 May (intact)	0.58 (0.505-0.666) n=21	1.47 (1.01-2.14)	
15 May (dissoc.)	0.95 (0.55-1.64) n=15	1.99 (0.95-4.13)	0.129 (0.145) n=28
22 May (intact)	1.02 (0.78-1.33) n=17	5.28 (2.01-13.88)	
22 May (dissoc.)	2.48 (0.89-6.9) n=8	9.42 (1.34-66.17)	0.681 (0.366) n=31

30 May (intact)	1.45 (0.82-2.56) n=20	2.68 (1.25-5.76)	
30 May (dissoc.)	1.61 (0.93-2.8) n=16	10.02 (2.33-43)	0.658 (0.37) n=12
6 June (intact)	0.102 (0.06-0.18) n=27	11.1 (4.31-28.57)	
6 June (dissoc.)	1.33 (0.82-2.15) n=23	26.45 (4.86-143.92)	0.444 (0.107) n=51

(A) 95% confidence interval calculated from the log normal distribution
(B) 95% confidence interval calculated from normal distribution

Dissociation constants throughout smoltification. Figures 12a and 12b respectively illustrate the dissociation constants (K_D) measured on non-dissociated (i.e., intact) and dissociated nuclear extracts obtained from salmon brains throughout the course of smolt transformation.

Throughout the course of smoltification, K_D from the dissociated preparations remains constant at approximately 1.0 nM, but there is a generally nonsignificant trend for the dissociated preparations to have reduced affinity relative to intact material from the same time period. (Eq. 3 and 5 explain that K_A, or binding affinity, is the inverse of K_D, the dissociation constant.) Binding affinity of the dissociated preparations illustrated by Figure 12b appears to decrease during 22 May and 29 April but the differences from other periods are not significant. In each of those cases the number of measurements was unusually small (df = 8 and 4 respectively).

After a significant decrease between mid-February and mid-March, the dissociation constants measured on the intact preparations illustrated in Figure 12a remain

constant at approximately 0.5 nM until 22 May when the binding affinity decreases significantly during the latter part of the serum thyroxine surge. During the last week of sampling, when the fish are considered downstream migrants, there is a significant increase in binding affinity in the intact nuclear extracts. The consistent increase in error on the determinations of "intact" K_D from 18 March through 30 May is not apparent in the dissociated preparations.

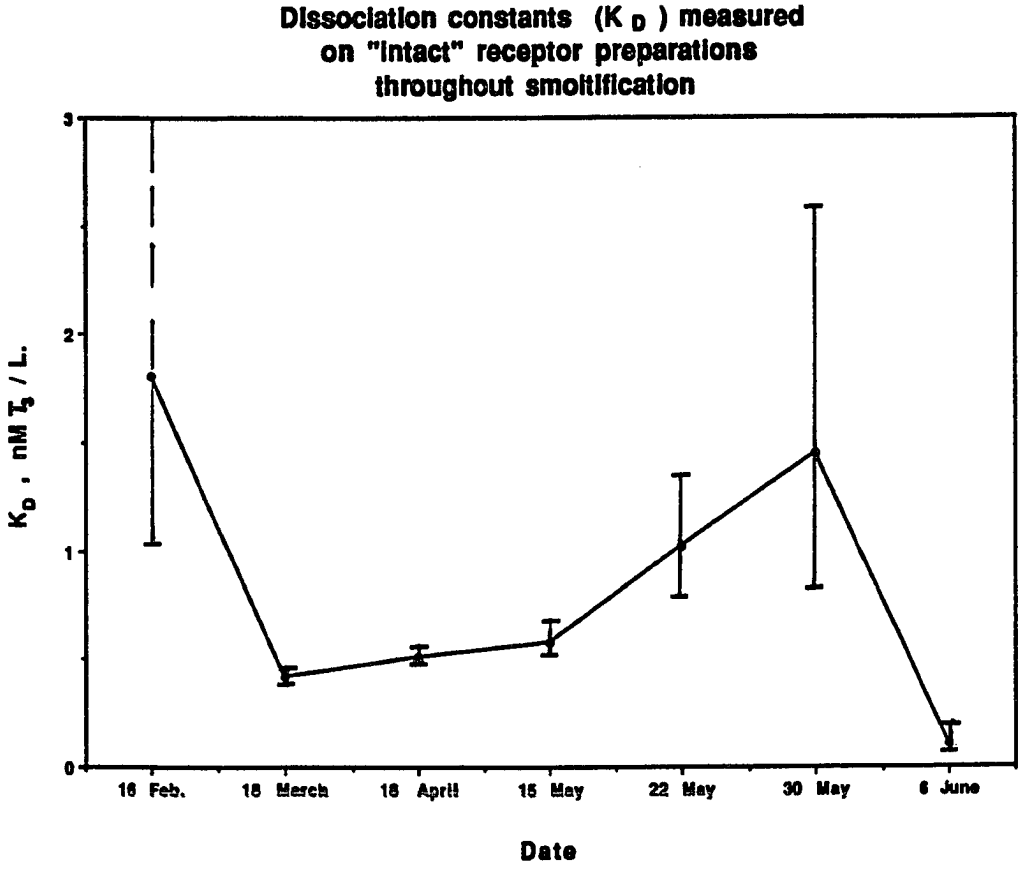


Figure 12a. Dissociation constants (K_D) obtained from homologous displacement experiments on "intact" brain nuclear extracts from throughout the sampling period. Intact extracts retain endogenously bound T_3 at the beginning of incubation. 95% confidence intervals were computed from the log-normal distribution.

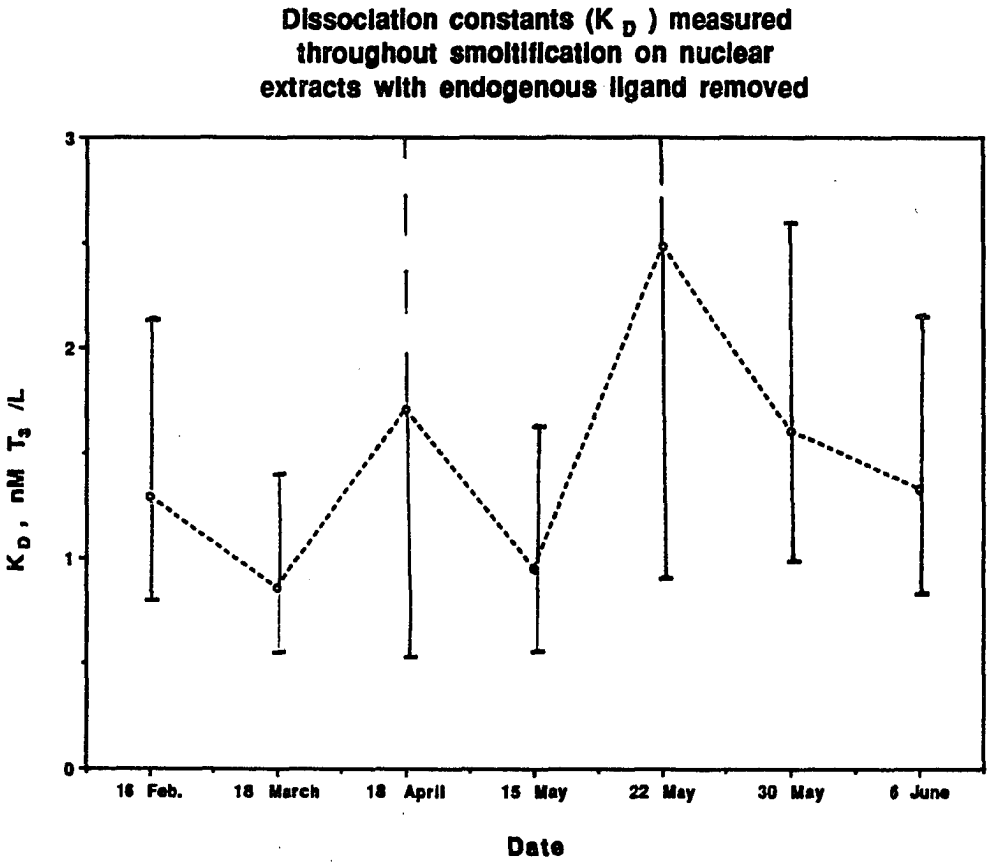


Figure 12b. Dissociation constants (K_D) measured from homologous displacement experiments on brain nuclear extracts with endogenously bound T_3 removed prior to incubation. These are called " dissociated extracts" in the text. 95% confidence intervals were computed from the log-normal distribution.

While there appear to be some changes in receptor behavior, especially that of the intact preparations, throughout the sampling period, the data failed to support the presence of multiple binding sites or cooperativity (Munson and Robard, 1980). The values for K_D appear within physiological range and consistent with literature values (Latham et al., 1976; Surks et al., 1975), although some workers have measured greater binding affinity (Bernal and Pekonen, 1984; Ferreiro et al.,

1987; Galton, 1988; Lee et al., 1990). Such differences may be related to different buffer conditions. Nonetheless, the data sugges that removal of endogenous ligand under this experimental protocol appears to have slightly altered the receptor.

Receptor concentration throughout smoltification. Figures 13a and 13b illustrate the binding capacity measured on nondissociated (intact) nuclear extract and the maximal binding capacity (MBC) measured on dissociated nuclear extracts throughout the sampling period, respectively. It is apparent the MBC tends to increase beginning 22 May and continues to increase until the migratory period, when the maximum concentration of receptors was measured. Discounting the results of 29 April, which had few determinations, maximal binding capacity of the dissociated receptor preparation during the migration period significantly exceeds that of similar preparations obtained on tissues sampled prior to May. A similar trend seems apparent in the binding capacity measured on the intact preparations, where the binding capacity of the migrant fish significantly exceeds that of 18 March and 15 May. The binding capacities measured 16 Feb and 29 April were, however, not signficantly different from either intact or dissociated preparations from the migrant period. In each of these cases the binding capacity of the intact preparations aberrantly exceeded that of the dissociated material from the same sampling periods, suggesting that receptor degradation may have occurred during the dissociation protocol. This observation introduces a need for prudence in declarations of significant differences in binding capacities between presmolt and smolted tissues.

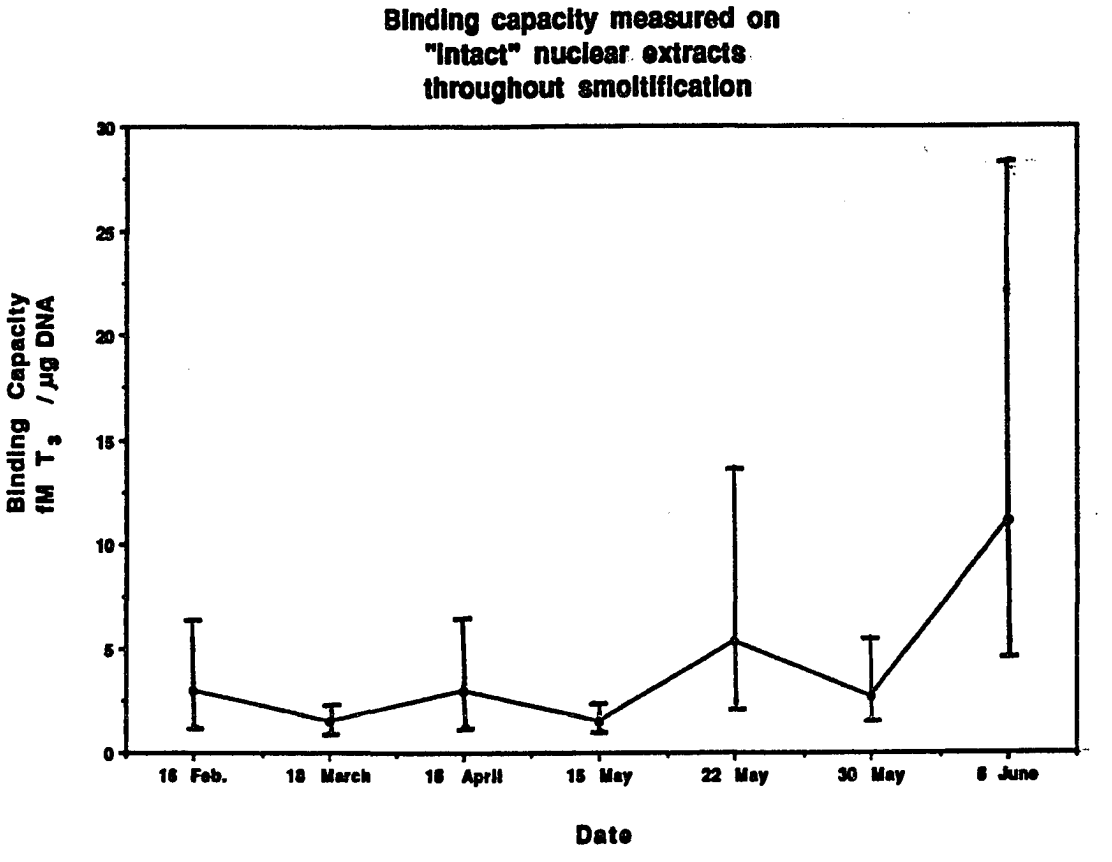


Figure 13a. Binding capacity determined from homologous dispacement experiments throughout the sampling period. Binding capacity is the receptor concentration per µg DNA extracted from preparations that retained endogenously bound T_3 at the beginning of incubation. 95% confidence intervals were computed from the log-normal distribution.

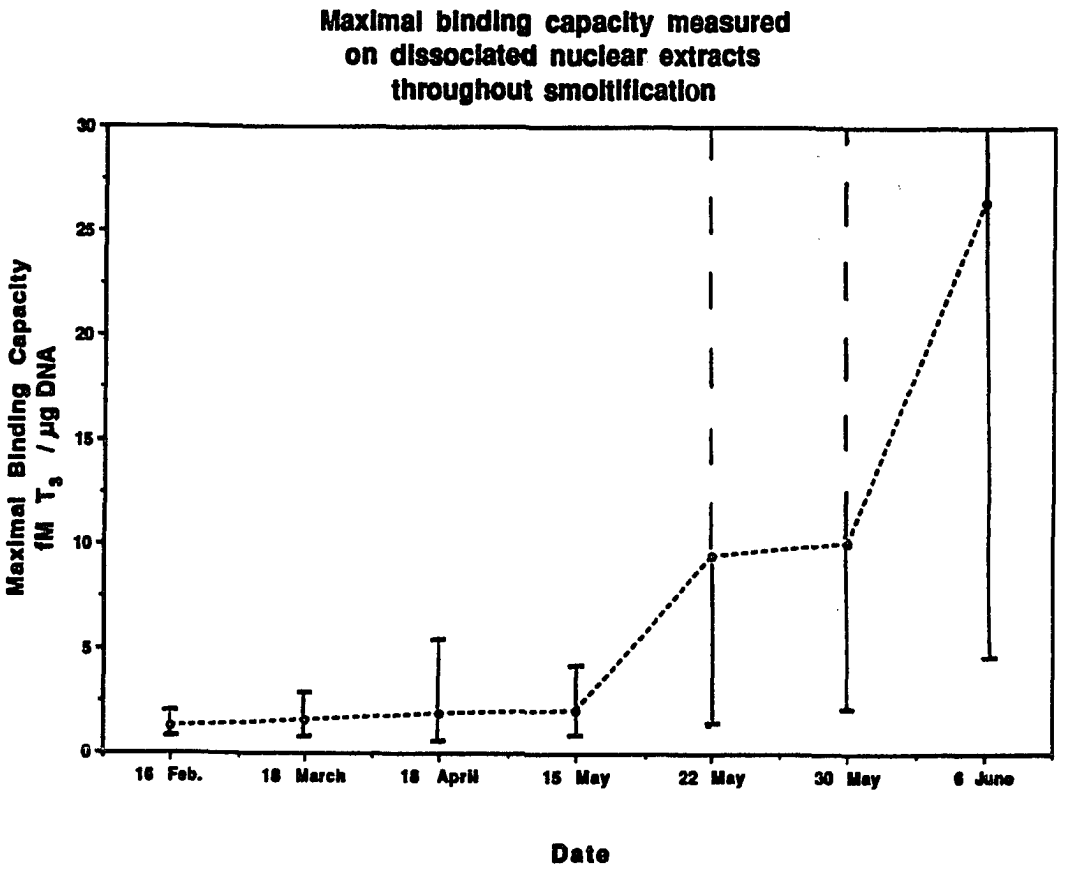


Figure 13b. Maximal binding capacity determined from homologous displacment experiments from tissues obtained throughout the sampling period. Maximal binding capacity is defined as the concentration of receptors per µg DNA extracted on preparations with endogenously bound T_3 removed by dissociation prior to incubation. Therefore, these preparations had the maximal number of receptor sites available for binding at the beginning of incubation.

Receptor saturation. Figure 14 illustrates the maximum probable percent binding by endogenous ligand calculated from the 95% confidence intervals of the binding capacities observed from intact and dissociated receptor preparations during each sampling period. The periods 16 Feb. and 29 April were considered indeterminate because the binding capacity measured on the intact preparations

exceeded that of the dissociated material. The percent endogenously bound is defined as the ratio of the difference of the maximal binding capacity of dissociated and minimal binding capacity of intact receptor preparation to the maximal binding capacity:

(% Endogenously bound= [(Diss B_{MAX} - Intact B_{MIN})/ Diss B_{MAX}] x 100). (16)

The result is conservative in that it uses the maximum probable difference between intact and dissociated preparations to determine the number of sites exposed by removing endogenous hormone. The figure suggests that the maximum probable occupancy of available sites by endogenous ligand increases substantially during the late smoltification and down-stream migration.

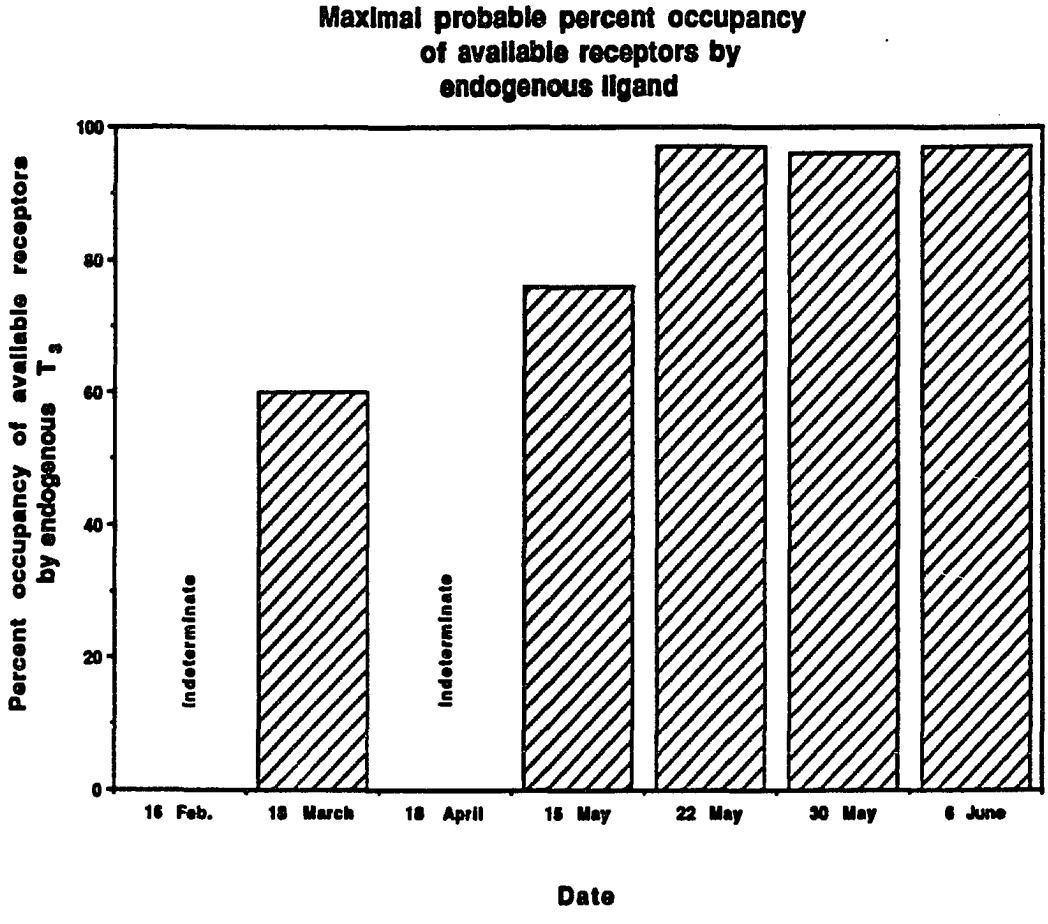


Figure 14. Maximal probable percent occupancy of available receptor sites by endogenous ligand. Endogenous ligand is T_3 bound to nuclear receptors at the time fish were sacrificed and which remained bound through the isolation protocol. The percent occupancy was calculated from % Occupied= [(Diss B_{MAX}- intactB_{MIN})/ Diss B_{MAX}] x 100, where Diss B_{MAX} is the maximum value of the 95% confidence interval for the maximal binding capacity from the dissociated preparations and intact B_{MIN}= the minimum value of the 95% confidence interval for the binding capacity from the intact preparations. The values for 16 Feb. and 29 April are indeterminate because the number of receptors measured from intact preparations exceeded that of the dissociated preparations for those sampling periods.

Dissociation rates. The dissociation rates of labeled receptor preparations measured at 4°C are illustrated in Figure 15 and listed in Table 1. Significant

differences occur between the periods before and after 15 May with the exception of 16 Feb. The significance of the Student's t interval was confirmed by merging the amount bound versus time regressions from the individual sampling periods with that from early June and subjecting the residual variances to an extra sum of squares F test. The rates measured from 22 and 30 May could not be distinguished from that of 6 June. Those measured from 15 March, 29 April, and 15 May had significantly different slopes and thereby slower dissociation rates. This is consistent with an increased number of receptors per μg DNA or a decreased dissociation rate constant (k_{-1}) during late smoltification. The F test also implied that the dissociation rate regression of 16 Feb. differs from measurements from late smoltification but no conclusion will be drawn because of large error in the rate determination from that period.

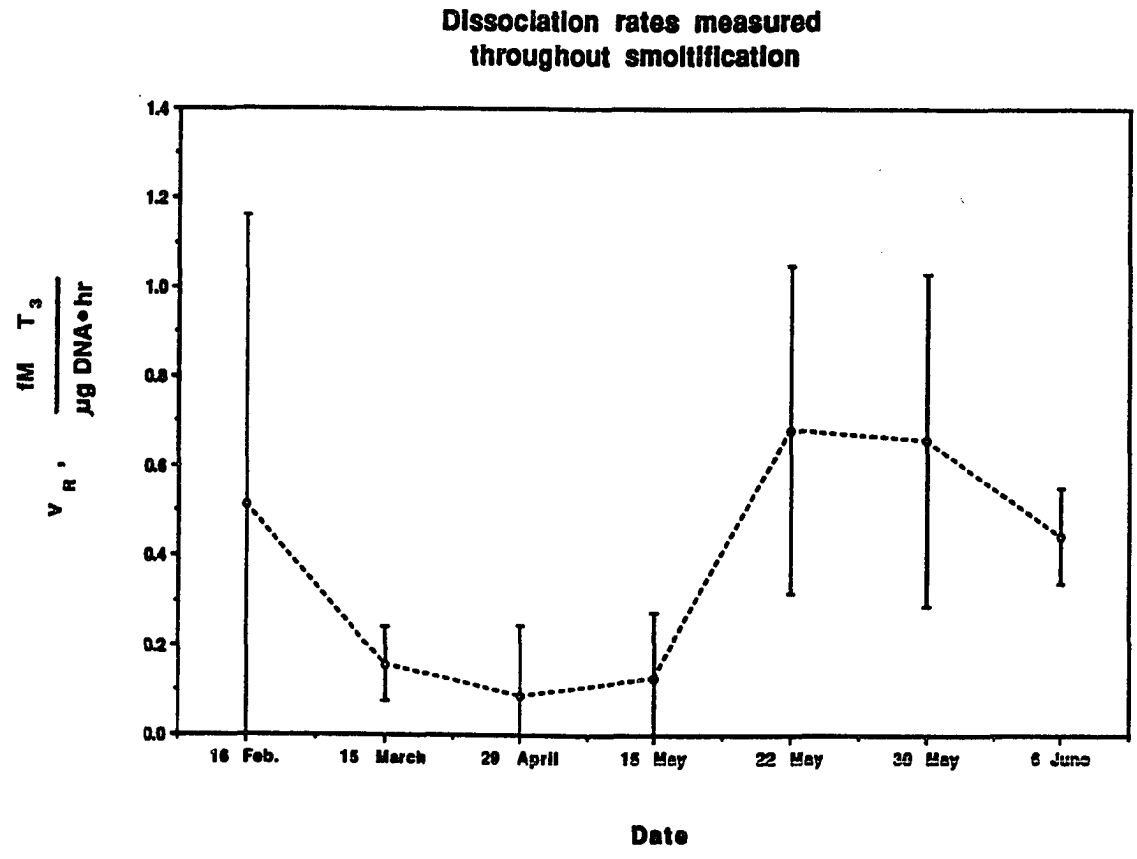


Figure 15. The rates of dissociation were measured by measuring the loss of radioactivity from labeled receptor over time at 4°C. Error bars are 95% confidence intervals.

The dissociation rate can be used to calculate the number of receptors exposed during a 24 hour incubation period. With the exception of the 6 June sampling period, the number of receptor sites that could be exposed by dissociation of endogenous ligand during a 24 hour incubation exceeds the maximal binding capacity. This result implies that all the sites occupied by endogenous ligand become available to labeled ligand during a 4°C incubation and confirms that equilibrium was established during incubation.

Rate Constants. The dissociation rate constants (k_{-1}) and association or binding rate constants (k_{+1}) calculated from Eqs. 2 and 3 are illustrated by the solid and dashed line, respectively, in Figure 16. The figure suggests that a change in rate constants occurs prior to smoltification: k_{-1} from 16 Feb is 27x greater than that of 6 June. However, the propagation of error over the course of several measurements prohibits any statement of significance except at the 80% level. A similar rationale influences statements concerning the apparent decline in association rate constants. Therefore, it is not possible to state with reasonable confidence that rate constants change during the sampling period with the present data.

The dissociation rate constants were anticipated to remain constant given the hypothesis that a single type of receptor dominated the population of available receptors during smoltification. Even though the kinetic experiments from individual time periods do not demonstrate that different receptors occur within a given preparation, the change in dissociation rates during late smoltification reinforces the notion that changes in the concentration of receptor coincide with the surge in serum thyroxine concentration. The dissociation constant (K_D) measured on intact tissues during the first week of June is signficantly different from other measurements indicating an increased binding affinity. Therefore, the possibility that the binding mechanism or the receptor itself is altered during late smoltification cannot be discounted. These data remain under analysis for the detection of changes in k_{-1} within individual determinations.

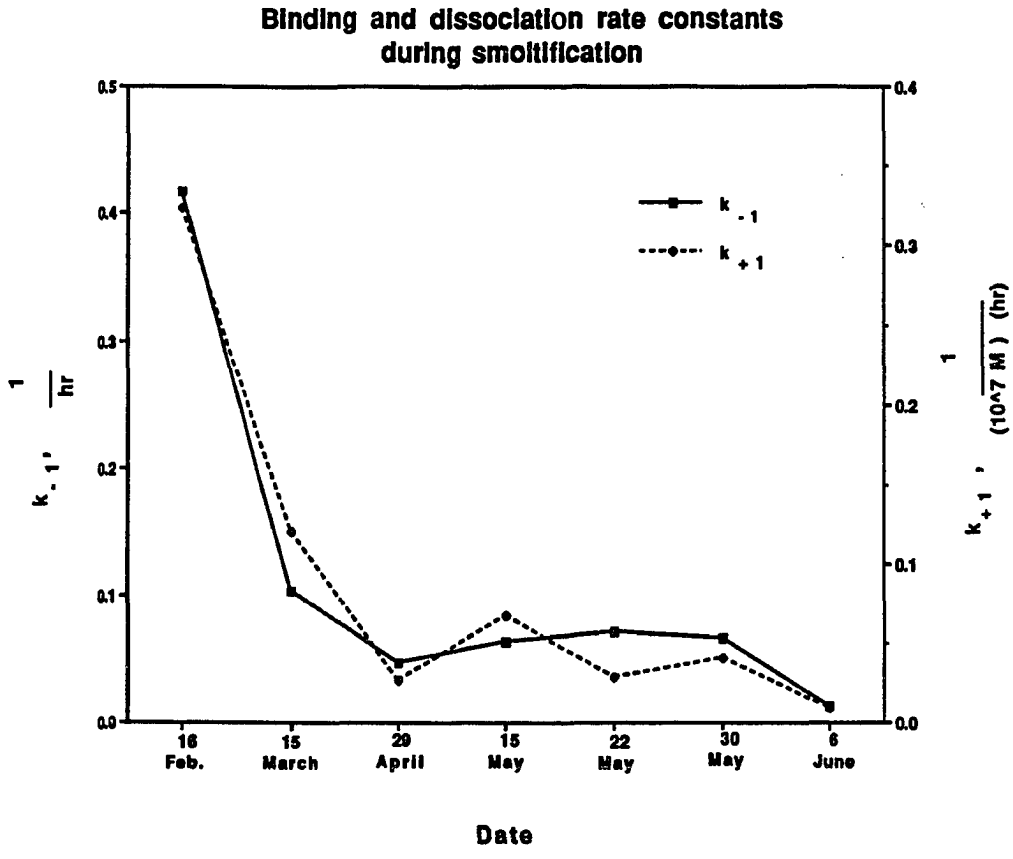


Figure 16. Association or binding rate constants (k_{+1}) and dissociation rate constants (k_{-1}) are designated as solid and dashed lines respectively. The values were obtained from the relation $V_R = (k_{-1})(nP_t)$, where V_R = dissociation velocities listed in Table 1 and (nP_t) = maximal binding capacity for the preparation from each sampling period, and from $K_D = (k_{-1})/(k_{+1})$. Mean values are illustrated because propagated errors from multiple determinations precludes statements of significance.

DISCUSSION

Thyroid hormone and smoltification. Serum thyroxine concentration undergoes a distinctive surge beginning in mid-May under the rearing conditions of this stock. The concentrations of thyroxine measured during 15 and 22 May are significantly greater than those measured prior to 29 April and compare favorably to serum T_4 concentrations reported previously for smolting salmonids (Dickoff et al., 1978; Grau, et al., 1982, 1985; Nagahama et al., 1982; Specker and Schreck, 1982; Sullivan et al., 1987). The abrupt decline similar to that which occurred 30 May has been reported, but not discussed, elsewhere (Grau et al., 1982; Grau et al., 1985; see also Cheek and Ebbesson, Addendum 1). As discussed below, such a change in serum thyroxine concentrations indicates either a decrease in thyroxine synthesis or an increase in iodothyronine accumulation in tissue depots (Specker et al., 1984). The spike that occurred 6 June was measured in fish retained by the hatchery after the remaining population had been released. The serum T_4 values from that time period were considered to be from out-migrating fish.

Under year to year conditions in a given hatchery, smoltification appears most intimately affected by temperature (Clarke et al., 1978; Grau, 1988; Wedemeyer et al., 1980) which usually varies dependent on solar heating (B. Och, Crooked Creek Hatchery, pers. comm.). Since development of the external characteristics of smoltification coincides with increasing thyroid activity (compare Figures 4 and 5 but see also Addendum 1) and thyroid activity is affected by temperature, it appears possible to monitor smoltification and detect abnormalities by measuring serum thyroxine in hatchery reared fish.

The serum thyroxine values for 30 May were low relative to 22 May and

6 June and not signficantly different from presmolt concentrations, suggesting a temporary abatement of thyroid activity or a rapid removal of hormone from circulation. In tadpoles the rate of T_4->T_3 conversion in the peripheral tissues is greatest during the metamorphic climax (Galton, 1988; Leloup and Buscaglia, 1980). Specker et al. (1984) argue that the rapid increase in circulating T_4 results from low T_4 tissue capacity before site specific changes induce increased capacity. Therefore, the sudden decline in serum T_4 concentrations that occurs during late smoltification may result from increased metabolism by tissue depots. Circulating T_4 values then increase dramatically and significantly during down-stream migration, consistent with high production and saturation of tissue depots.

Rationalizations for the surge in thyroxine concentration. These data do not address why the thyroxine surge occurs. To my knowledge, no simultaneous measurements of plasma TSH and TH have been made during smoltification. If euthyroid or greater concentrations of TSH persist in concert with increased plasma thyroxine, it suggests either that hypothalamic TRH is stimulating the surge, that the negative feedback of T_3 on TSH beta subunit transcription is not operative (Gurr et al., 1990; Wondisford et al., 1989), or that the thyroid gland becomes hypersensitive to TSH (Specker et al., 1984). Alternatively, non-thyroidal mechanisms, such as increased plasma prostaglandins, may participate in the enhanced thyroid activity (Takasu et al., 1982). I am not aware of any measurements of plasma prostaglandins in salmon, but in view of the increased fragility of fish tissues during smoltification (discussed below), such measurements may be worthwhile.

Seasonal periodicity in thyroid activity (Grau, 1988) suggests that the

hypothalamic-pituitary-thyroid axis is subject to photoperiod control of circadian rhythms, i.e., suprahypothalamic influence. This concept is supported by the high protein synthesis and large density of T_3 receptors observed in the pineal gland during smoltification (Ebbesson et al., 1990). In any respect, these speculations emphasize that present notions of feedback homeostatic control of thyroid function are challenged by the surge in serum thyroxine during smoltification.

In vivo response and thyroxine concentration. Figure 5 illustrates the change in the appearance of the fish as subjectively measured during sacrifice. This numerical identification of "stage" is similar in concept to that used to identify the development of metamorphosis in tadpoles (Kollros, 1961). While the distinction between pre-smolts (stage 1) and smolts (stage 4) is apparent to even a casual observer, separation of the intermediate stages is primarily dependent on the loss of pigment spots on the belly and flanks and the gradual occulsion of parr marks (see Addendum 1). Between mid-April and mid-May, the period representing the boundary between normal and hyperthyroid conditions, the percentage of fish showing a loss of pigment spots increases. By 22 May no distinctive stage 1 presmolts were observed and by late May the majority were stage 4 smolts but were not salt water tolerant (B. Och, pers. comm.) The fish were released 3 June, about 2 weeks earlier than normal, because an unseasonal increase in water temperature (from 9°C to 12°C) induced agitated swimming, crowding in the lower raceway, and subsequent oxygen depletion. A comparison of Figures 5 and 4 would suggest a relation between the increases in serum thyroxine and the external indices of smolt transformation. However, it is noteworthy that the appearance of silvering preceeds the increase in T_3 receptor concentrations in the

brain (Figures 13 a and b) suggesting that if silvering results from thyroid hormone action as proposed by some workers (Miwa and Inui, 1985), then such a response in vivo may have a lower threshold for thyroid hormone concentrations than hormone-influenced changes in the brain. (See also Addendum 1 for further discussion of this point.)

Nuclear fragility. Successful completion of this investigation was delayed for several years because I was unable to isolate brain nuclei using a standard method of centrifugation through a dense sucrose solution (Eberhardt, 1978; Valcana and Timeras, 1978). When the pellet containing nuclei was transfered to buffer, the nuclei would invariably burst resulting in an agglutinous mass containing little if any DNA. The pellet failed to demonstate any specific binding of T_3. This phenomenon of increased nuclear membrane fragility appears similar to that observed in neonatal rat tissue (DeGroot et al., 1977) and may be related to the increased "leakage" of receptor from the nuclei (Bernal et al., 1978). C.V. Sullivan (pers. comm.) also experienced difficulty isolating nuclei from smolting salmon. Increased tissue fragility during smoltification appears to be an uninvestigated issue even though scale loss in smolts is a common source of mortality in hatchery reared salmon. Hatchery workers, who handle the fish throughout culture, describe smolts as feeling "soft, like a month-old pickle" as opposed to a firm, fresh one. Several years of disappointing failure suggested to me that changes in cell membranes occur prior to the onset of observable hormonal or gross physical changes associated with the smolt metamorphosis.

In desperation I attempted to stabilize the nuclear membrane by adding 20% glycerol and 1 mM $CaCl_2$ to the homogenization medium. This serendipitous

addition was successful and I was able to recover brain nuclei throughout smolt transformation. It has been stated that yields of nuclei from homogenate of less than 60% indicates that a subpopulation of nuclei is selected by the isolation protocol (Bellard et al., 1989). Using the SGC isolation protocol, an abrupt change in nuclear yields occurs in mid-April from about 70% to less than 30% (Figure 6), suggesting that the nuclei used throughout the later part of this investigation represent a subpopulation of the whole. Furthermore, in spite of an apparent generalized fragility, as smoltification progressed the isolated nuclear pellet became more refractory to perchloric acid digestion and required prior treatment with high salt concentrations before the chromatin could be oxidized for the analysis of DNA by the Burton method. Together these observations suggest that some change occurs in the nuclear and perhaps other membranes prior to and during smoltification.

A membrane bound transporter? Unfortunately the isolated nuclei failed to reproducibly demonstrate specific binding of T_3. Figure 7 illustrates that maximum binding was observed only in the salt extract of nuclei and not in the intact nuclear pellet. The highest concentration of KCl used, 0.4M, is the same used to extract nuclear protein and has been used many times during purification of T_3 and other nuclear receptors (DeGroot et al., 1974; Samuels et al., 1974; Surks et al., 1973). When the nuclei were incubated in a medium containing 0.2 M KCl, the nuclei visibly degraded and the maximum binding by the pellet was observed. However, 0.2M KCl is probably insufficent to solubilize all the nuclear protein, so the radioactivity retained by the pellet is likely due to chromatin exposed to solution by degradation of the membrane. Only when the nuclear proteins were

thoroughly solubilized by salt extraction were substantial amounts of radioactivity retained by the ultrafilters. This observation compelled me to abandon attempts to measure specific binding on nuclei; consequently, all binding studies were carried out on nuclear proteins extracted by 0.4 M KCl.

This experiment also suggested that some component of the normal nuclear membrane was deleted during isolation. The buffered triton wash immediately after SGC sedimentation removes the outer nuclear membrane (Blobel and Potter, 1966; DeGroot et al., 1974; Surks et al., 1973). Kastellakis and Valcana (1989) recently demonstrated a Na^+ dependent thyroid hormone transporter in the plasma membrane. In addition, Oppenheimer and Schwartz (1985) have described a stereospecific pump that concentrates T_3 in the nuclei. Specific binding of TH has been observed in salmon brains by in vivo methods (Scholtz et al., 1985). I observed no reproducible specific binding of T_3 by isolated nuclei but have observed specific binding by the proteinaceous receptor extracted from the nuclei with 0.4M KCl. It appears likely that a thryoid hormone transporter exists in the outer nuclear membrane of salmon brains.

T_3 binding behavior throughout smoltification. The results of the homologous displacement experiments suggest that there may be subtle changes in the dissociation constant throughout the sampling period especially during the migrant phase. The results are not qualitatively inconsistent with determinations of K_D made on metamorphosing tadpole red blood cells (Galton, 1988) and during the mammalian neonatal period (Lee et al., 1990; Ishiguro et al., 1980). Interestingly, the lowest binding affinity measured after 18 March in intact nuclear extract occurred at the same period as the sharp reduction in circulating thyroxine,

while the greatest affinity occurred just one week later during the migrant stage. These data suggest that a change in the nature of the receptor occurs rather suddenly during the final stages of fresh water residence in smolting coho salmon.

Determinations of dissociation rate constants have been used to demonstrate the presence of multiple binding processes occuring within a given mixture (Mayo et al., 1989). Changes in the dissociation rate constant throughout smoltification would suggest a change in receptor strategy, i.e. a change in the receptor itself or a change in the type of receptor. As seen in Figure 16, the rate constants appear to change throughout the sampling period but remain more or less constant after 29 April. Such an observation would be consistent with the changes in receptor type that are believed to occur in mammalian brain during gestation and the neonatal period (Strait et al., 1990). However,the amount of propagated error from multiple determinations precludes any statement of the magnitude or significance of the observed changes. These data remain under analysis. Determinations of possible changes in receptor type can be accessed directly, however, by changes in electrophoretic mobilities of the nuclear extracts.

The dissociation of endogenous hormone at warmer temperatures reduced binding affinity in previous investigations (Ferreiro, 1990; Lee et al., 1990). With nuclear extracts of this study, K_D of the dissociated preparations tend to be equal to or slightly greater than those measured from the intact preparations, the differences being significant for the migrating smolts. The affinity of dissociated receptor preparations does not change significantly throughout the sampling period. While the data do not allow a broader interpretation, the act of dissociating endogenous ligand at warmer temperatures may have affected the nature of the receptor.

Receptor concentration throughout smoltification. The maximal binding capacity increased after mid-May and was greatest during the period of out-migration. The increase in receptor concentration lagged the initial surge in serum thyroxine by one week. This result suggests that, similar to the increase in tadpole beta receptors (Yaoita et al., 1990) during metamorphosis, beta receptors in chick brain (Forrest et al., 1991), pituitary beta-1 receptors in rat (Hodin et al., 1990), and the regulation of brain nuclear receptors in fetal, neonatal or hypothyroid mammals (Dozin and De Nayer, 1984; Perez-Castillo et al., 1985), that the number of thyroid hormone receptors in the brains of smolting salmon changes with the changing hormonal regime. Futhermore, the results are consistent with Specker et al. (1984) who demonstrated that tissue capacity for iodothyronines increased a little later than the initial increase of serum thyroxine in smolting salmon.

The observation that smoltification is accompanied by an increase in the number of receptor sites is confirmed by an independent set of experiments. Figure 15 illustrates that a significant difference in dissociation rates occurred between 15 and 22 May within the same time frame as the observed increase in MBC. Assuming that the dissociation rate constant does not vary throughout late April, May, and June, the differences in dissociation velocity suggest that late smolts contained from 3 to 5 times more receptors per μg DNA than did pre-smolts, or a little less than a similar ratio determined from the MBC.

Receptor concentration and the isolation protocol. The receptor concentrations measured towards the smolt transformation climax are appreciably greater than previously observed in non-smolting salmon tissues (Bres and Eales,

1986; 1990; Darling et al., 1982), metamorphosing tadpole (Galton, 1988), or the fetal or neonatal rat (Ferreiro et al., 1987; Ishiguro et al., 1980). However, receptor concentrations measured in pre-smolt brains are not dissimilar to those observed in purified neuronal nuclei from adult rat neocortex (Gullo et al., 1987), rat fetal brain (Perez-Castillo et al., 1985) or human fetal brain at 16-18 weeks (Bernal and Pekonen, 1984).

The high receptor concentrations during late smoltification may be attributed to two factors: 1) an absolute increase in receptor number in particular cell types coupled with 2) nuclear selection by the isolation protocol. Further, since the olfactory bulb is known to increase in volume during smoltification (Ebbesson et al., 1990), regional concentrations of receptors (Gullo,1990) could increase the apparent total brain concentration of receptors by cell division. As illustrated by Figure 6, an abrupt change in the yields of nuclear DNA from tissue homogenate occurs beginning in mid-April. As discussed above, this may be related to changes in the physical structure of tissue membranes and the reduced recoveries suggest that selection may have occurred (Bellard, 1989). Since a limited survey of T_3 binding of other fractions of the isolation protocol did not suggest the presence of specific binding (data not shown), the isolation procedure may have inadvertently selected for those nuclei that possessed thyroid hormone receptors. If such were the case, then the 10 fM T_3/μg DNA MBC observed from tissues of 30 May may represent about 1.8 fM T_3/ μg DNA in the brain as a whole. This concentration compares favorably to the MBC of rat pituitary nuclear receptors (1.5 fM T_3/μg DNA) (Larsen and Silva, 1983), rat brain receptors 30 days post partum (1.0 fM T_3/ μg DNA) (Ferreiro et al., 1990), or purified adult rat neocortical nuclei (1.6 fM

T_3/µg DNA) (Gullo et al., 1990). However, when adjusted for nuclear percent yield, the migrating smolts MBC of 26 fM T_3/µg DNA remains quite high at 6.3 fM T_3/µg whole brain DNA. If the MBC is corrected, assuming selection of T_3 receptor-containing nuclei by the isolation protocol, whole brain concentrations of receptors increase according to the trend illustrated in Figure 17. This assessment of the data suggests that nuclei containing thyroid hormone receptors possess physical characteristics that permit their selective isolation using the protocol described under Methods. Further, the data indicate an overall increase in brain nuclear receptors during smoltification.

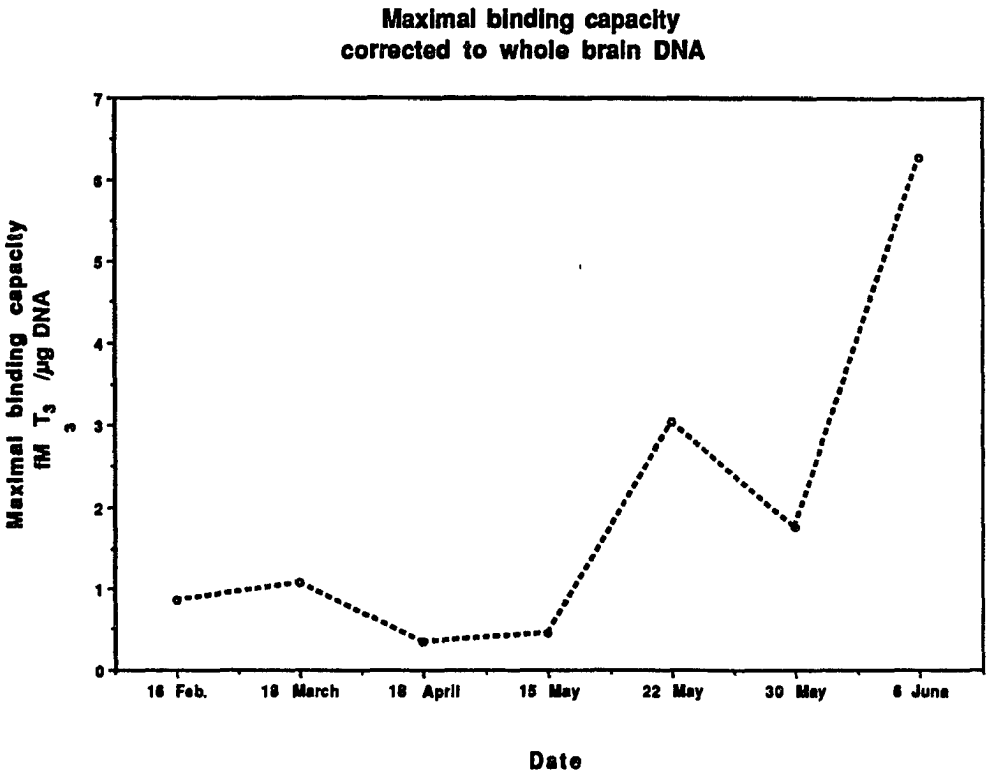


Figure 17. The T_3 maximal binding capacity of whole brain DNA. The maximal binding capacity was adjusted for losses of DNA during the isolation protocol assuming that the methodology favored the survival of those nuclei that contained T_3 receptors.

Receptor saturation. Not only did the number of receptors increase, but the maximal probable saturation of available receptors by endogenous hormone increased, beginning in mid-May parallel to the increased availability of hormone. The maximum probable percent occupancy of available receptors was calculated from the extrema of 95% confidence intervals for binding capacity of intact and dissociated preparations. This calculation is conservative in that it estimates the greatest probable number of sites exposed by dissociation and thereby the largest percent of the sites already occupied by endogenous ligand in the intact preparation. Therefore, the calculations of maximum probable percent occupancy illustrated in Figure 14 likely overestimate the percent occupancy in vivo. Other investigations report endogenous percent occupancies from 30-90% based on the ratios of the mean values of maximal binding capacity of dissociated and intact receptor preparations (Crantz et al., 1982; Ferreiro et al., 1990; Lee et al., 1990). Calculated this way, the percent occupancy by endogenous ligand during smoltification ranges from 5% on 18 March to 58% 6 June. Since biochemical response is a function of the percent saturation (Halperin et al., 1990), the increased receptor saturation during late smoltification implies that a different cadre of transcriptional products are present.

Intranuclear T_3 concentration. The percent occupancy could be determined directly if a reliable estimate of the intracellular concentration of T_3 were available. This is not possible because any homogenate of brain tissue would be contaminated by hormone circulating in blood and cerebral spinal fluid. Furthermore, T_3 is concentrated in nuclei relative to cytoplasm (Refetoff and Larsen, 1989). However, the concentration of hormone available to receptor can be

calculated indirectly from the Michaelis-Menton equation. The intranuclear concentrations of T_3 illustrated by Figure 18 were calculated from the K_D measured in the dissociated preparation and where v/V_{max}= maximal probable percent occupancy. (The use of maximal probable percent occupancy is relevant here because it is an estimate of V/V_{max} in situ.) The values range from 1.3 nM in presmolts to 80 nM during 22 May and decline slightly thereafter. T_3 is concentrated in cytoplasm relative to serum by about 3 times, but the source of most cytoplasmic T_3 in the brain is local deiodination of T_4 (Crantz and Larsen, 1980). Further, a stereospecific pump within the nuclear membrane increases the concentration of T_3in the nuclei 50-200 fold over that in the cytoplasm (Oppenheimer and Schwartz, 1985). Assuming a plasma T_3 concentration of 1.5 nm (Dickoff et al., 1985), even the highest concentration of nuclear T_3 is well within reasonable expectations. These calculations support the contention that local deiodination of T_4 increases during smoltification similar to that observed in the metamorphosing tadpole (Galton, 1988; Leloup and Buscaglia, 1980).

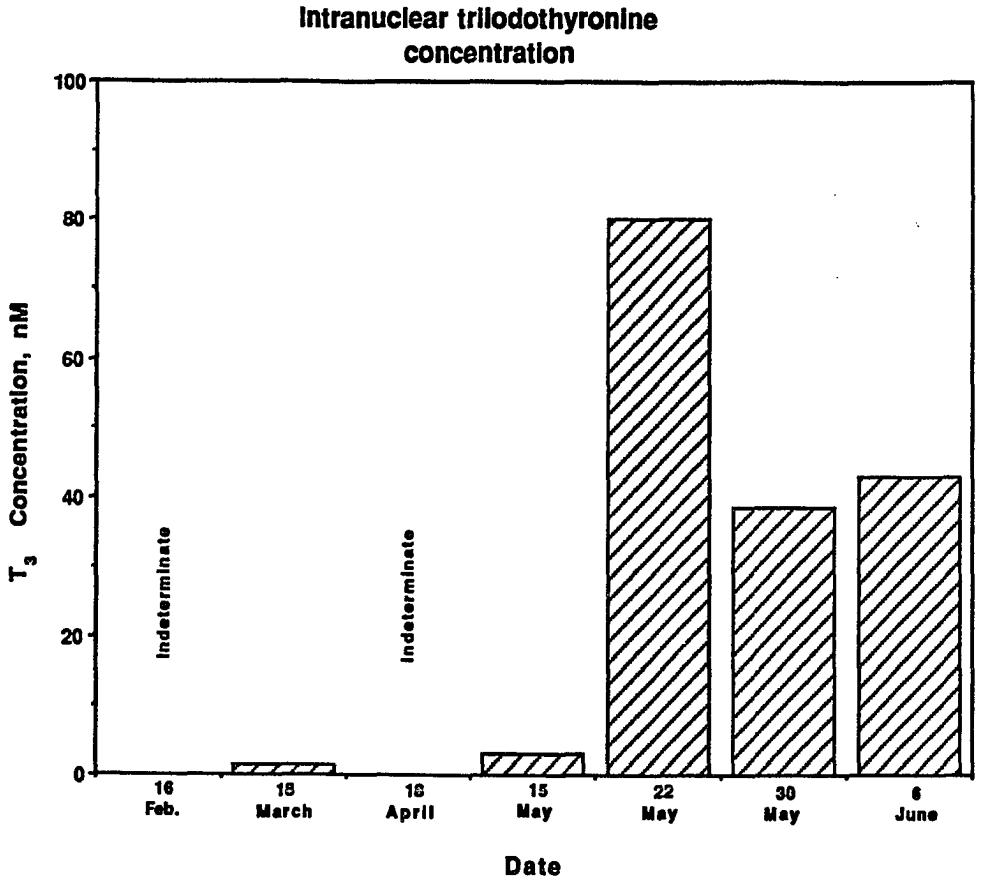


Figure 18. The calculated intranuclear T_3 concentration at the time fish were sacrificed. The values were obtained from the Michaelis-Menton equation, $v/V_{max} = [S]/ K_D + [S]$, where v/V_{max} is the maximal probable percent binding. The maximal probable percent occupancy is directly comparable to v/V_{max} because it is an estimate of endogenously bound/ maximal binding.

THYROID HORMONE AND THE SALMONID OLFACTORY IMPRINT

The increase in the number of TH receptor sites begins to occur during mid-May, slightly later than the increase of circulating thyroxine. This period coincides with changes in the whole brain concentrations of dopamine, serotonin, gamma aminobutyric acid, and other amino acid neurotransmitters (Ebbesson et al., 1987; Ebbesson et al., 1989 a,c ; Ebbesson et al., unpublished).

The similarity of changes in the brains of smolting salmon to the reorganization of the brain that accompanies neonatal development or amphibian metamorphosis prompted Ebbesson and Bazer (1990) to propose that salmon smoltification is a "mid-life neuroembryonic period." In previous work, primarily on mammals, thyroid hormone has been shown to participate in a vast and somewhat confusing array of biochemical activities. These result in the proliferation of nerve fibers and, later, in the sculpturing effect of cell and fiber death that eventually results in a reorganization of the brain. An attractive hypothesis is that thyroid hormone receptor, once occupied by ligand, regulates the transcription of growth factor and other hormones with pleiotrophic effects (Fisher et al., 1982). The discovery that the different thyroid hormone receptors are ontogenetically and differentially expressed in brain tissue (Cook and Koenig, 1990; Forrest et al., 1990; 1991; Hodin et al., 1990; Strait et al., 1990) leads further to the hypothesis that each member of the thyroid hormone receptor family has specific transcriptional effects, some of which are only active during development in particular portions of the brain.

Substantial and long-lasting behavioral alternations including olfactory imprinting are constitutive components of late smoltification. Since the increase

in maximal thyroid hormone binding capacity during late smoltification is not unlike that observed in neonatal mammals or metamorphosing tadpole, and in view of the dependence of neurological development of other vertebrates on the actions of thyroid hormone, the data presented here indicate that thyroid hormone is intimately involved in the reorganization of salmon brain that occurs as a prerequisite to olfactory imprinting.

ADDENDUM 1

GOITROGENIC EFFECTS OF PROPYLTHIOURACIL DURING SMOLTIFICATION IN COHO SALMON (ONCORHYNCHUS KITUSCH)**

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Juvenile Coho salmon undergo a metamorphosis called smoltification prior to entering salt water. An increase in serum thyroxine concentrations occurs during the same period prompting the hypothesis that thyroxine controls some aspects of the metamorphic process.

Coho salmon presmolts were reared under two different concentrations of the goitrogen, 6 n propyl 2 thiouracil (PTU). Measurements of serum thyroxine, appearance, and salt water tolerance were performed to assay the effects of hypothyroidism throughout smoltification.

Fish reared in 10 mg/l. PTU did not demonstrate the characteristic surge in serum thyroxine concentrations but otherwise appeared able to osmoregulate and complete smolt transformation equally well as controls. Fish reared in 25 mg/l PTU showed a rapid decline in thyroxine concentrations and failed salt water challenge. The results suggest that some aspects of smoltification may be arrested or reversed by anti-thyroid compounds.

** For submission to Aquaculture

Introduction

Salmon culturalists use "smoltification indices" to determine the appropriate time to release salmon smolts into the marine environment. A number of readily observable smoltification indices have been described (Folmar and Dickoff, 1980); namely, parr marks disappear, the fish becomes silvery, condition factor changes to a more streamlined configuration (Hoar, 1976) and the fish starts to actively move downstream (Zaugg, 1983). Less obvious, complex physiological metamorphoses preparatory to marine life have been studied in recent years, i.e., hemoglobin changes to an adult form (Sullivan et al., 1985) and overall metabolism increases (Higgins, 1985). Increased lipolytic rates (Sheridan et al., 1985) are accompanied by decreased fat and glycogen in liver and muscle (Woo et al., 1978). The salt water challenge test commonly used in hatcheries to predict marine survival is based on changes in gill Na^+K^+ATPase activity (Folmar and Dickoff, 1981; Clarke et al., 1978) and osmoregulatory capacity (Nagahama et al., 1982). The olfactory imprint that will later guide the mature fish back to the spawning grounds occurs at this time (Hasler et al., 1978). Phases of the moon, day length, and water temperature (Grau et al., 1982) have been correlated with downstream migration and salt water tolerance.

Plasma concentrations of thyroxine increase substantially during smoltification, an observation prompting some researchers to predict that thyroid hormone participates in smoltification much like thyroxine controls metamorphosis in amphibians (Dickoff et al., 1978). Since amphibian metamorphosis can be arrested with goitrogens (Kollros, 1961), it may be possible to attenuate thyroid hormone dependent processes during smoltification using antithyroid compounds.

Goitrogens are commonly used to induce experimental hypothyroidism in laboratory animals, but the affects of goitrogens on smolting salmon have not been clearly established. Baggerman (1961) reversed salt water preference in chinook smolts following exposure to thiocyanate, but thiocyanate failed to significantly lower plasma thyroxine concentrations or affect radioiodine incorporation in adult rainbow trout (Eales and Shostak, 1983). Thiourea treated amago salmon smolts did not alter carbohydrate metabolism (Miwa and Inui, 1983). Propylthiouracil (PTU) inhibited smolt-like appearance and the formation of adult forms of hemoglobin in a dose-dependent manner when fed to smolting coho salmon (Sullivan et al., 1985) but did not prevent the thyroxine surge (Sullivan, 1986).

Even though propylthiouracil affects thyroidal biosynthesis (Greer et al., 1964), plasma concentrations of T_4 may not be affected because of inhibition of deiodination in target tissue (Escobar and Del Ray, 1967). Sullivan (1986) suggests that PTU does not affect thyroidal synthesis or metabolism of T_4 in salmon smolts but may affect the number of hormone receptor sites in competent tissue.

Our research is to define the role of thyroid hormones in smolt transformation and imprinting. This work investigates the role thyroid hormone may play in salmon metamorphosis by treating smolting salmon with goitrogens and measuring changes in salt water survival, plasma thyroxine, and readily observed morphological characteristics.

We report here two experiments; one, to determine the effect of PTU on plasma T_4 concentrations and smolt transformation in a population of coho salmon under laboratory conditions and two, to determine if plasma T_4 levels and smolt transformation could be altered by PTU in the middle of the process.

Materials and Methods

Experiment A

Approximately 1000 coho parr were received from the Crooked Creek Hatchery, Soldotna, Alaska, in late March. They were divided into groups that received either PTU or no treatment. Lighting was supplied with total spectrum fluorescent lamps activated with timers set to ambient light conditions. Charcoal filtered domestic water was aerated amd cooled in an elevated resevoir and the tanks supplied by gravity feed. Water temperature, flows, and goitrogen delivery were monitored daily. PTU treatment began 15 April and concentrations were maintained at approximately 15 ppm until 7 June when the experiment was terminated.

During Experiment A, as each fish was weighed and measured, it was examined for external signs of smoltification and assigned a value of 1 to 5 which was called "stage" of smoltification. The numerical values described below are an expansion of the 3 stage criteria used by Scholz (1983) and are applicable to fish reared in clear water under well lighted conditions.

Criteria for Determining Smoltification Stage

Stage 1

The fresh water phase or parr has distinct, vivid parr marks. Small melanophores occur laterally and on the belly. The dorsum is olive-green.

Stage 2

Ventral melanophores become faint or not apparent leaving the belly white. The parr marks below the lateral line appear to be fading but

remain discernable.

Stage 3

The parr marks ventral to the lateral line are less apparent. The parr mark nearest the fork of the tail is fading or not apparent. Enhanced pigmentation can be observed on the tips of the dorsal and tail fins.

Stage 4

In the smolt the anterior and posterior parr marks are faint. The dorsum becomes bluish-green or lime green. Occasionally, blue iridescent pigmentation can be seen on the dorsal surface of the eye. The cap of pigment over the optic tectum may acquire a blue iridescence. Palpebrae are obvious. Scales are readily lost. Fish reared in attenuated light or opaque water may retain a darker overall appearance but will acquire numerous silvery scales.

Stage 5

Stage 4 fish that have survived 3 days in salt water.

Experiment B

Approximately 1000 coho presmolts at apparent stage 3 were obtained from the Ship Creek Fish Hatchery, Anchorage, Alaska, in late April. These fish originated from the Bear Lake 1985 brood year and had been reared in water 7.2 to 7.7°C since 5 Jan., as part of an accelerated rearing program. They were transported to Seward in aerated tanks held at ice water temperatures and immediately divided into groups. The fiberglass tanks were kept outdoors, but shaded with plywood, and, as in Experiment A, were supplied with charcoal filtered domestic water that had been cooled in an elevated resevoir. Each tank received aeration.

Beginning 27 April, one of the tanks received a drip of propylthiouracil (PTU) such that the final concentration of goitrogen was 20-25 ppm. The experiment was terminated on 3 June. Fish were fed ad lib with OMP generously donated by the hatchery. The stage of smoltification was not determined as these fish appeared to be advanced smolts when received.

One experimental group of 60 stage 1 fish that had been maintained at 0-3°C was obtained from the Crooked Creek Hatchery, Soldotna, Alaska, on 12 May and processed immediately.

Thyroxine Radioimmunoassay.

Fish were collected at 7-10 day intervals from the control tank throughout the smoltification period. Fish from the PTU tank were collected more infrequently. A minimum of 30 specimens per time period were anaesthesized in MS222, blot dried, and weighed and measured to determine condition factor (Virtanen and Soivio, 1985). The tail was severed and blood collected from the caudal stump with heparinized Natelson blood collection capillaries. Blood from either 3 (exp. A) or 5 (exp. B) individuals was pooled in 1.5 ml centrifuge tubes and centrifuged at 10,000 g for 3 minutes. The resultant plasma was pipeted and frozen until analysis. Thyroxine was determined by RIA using a kit developed for analysis of human serum (BioRad, Quantaphase). Reconstituted normal horse serum (Sigma) was used as an interassay standard. Because fish in these tanks were the source of specimens for other experiments being conducted simultaneously (data not discussed), blood plasma from additional individuals was obtained at irreglular intervals.

Salt water challenge.

At selected times late in the smoltification cycle, 6 fish from each experimental tank were transfered to full strength sea water. Mortalities were determined after 3 days. Sluggish or apathetic behavior at the end of three days was considered mortality because our experience indicated that such individuals die soon. Dying animals usually became pigmented and appeared to darken relative to their fresh water appearance. Three days was chosen because plasma cortisol reaches maximum concentrations in about 72 hours after salt water entry (Forrest, et al., 1973) and we felt that a more realistic test should include the time frame of response to osmoregulatory stress.

Condition factor.

Condition factor was calculated according to Virtanen and Soivio (1985) where C.F. = 100 x weight (gs.)/ length (cm.)^3.

Data were subjected to ANOVA statistics and the factor level means (both treatment and date) tested for significance using the t-interval.

Results

Experiment A

PTU delivery began 13 April and was terminated 7 June. Goitrogen concentrations stabilized to approximately 10 mg/l after early May (Figure 1A). Water temperatures are illustrated in Figure 2A. For approximately a 2 week period in early May, water temperatures in the treated tank were 1-2°C warmer than the control tank and could not be cooled further because of the reduced flows required to deliver an economic dose of goitrogen.

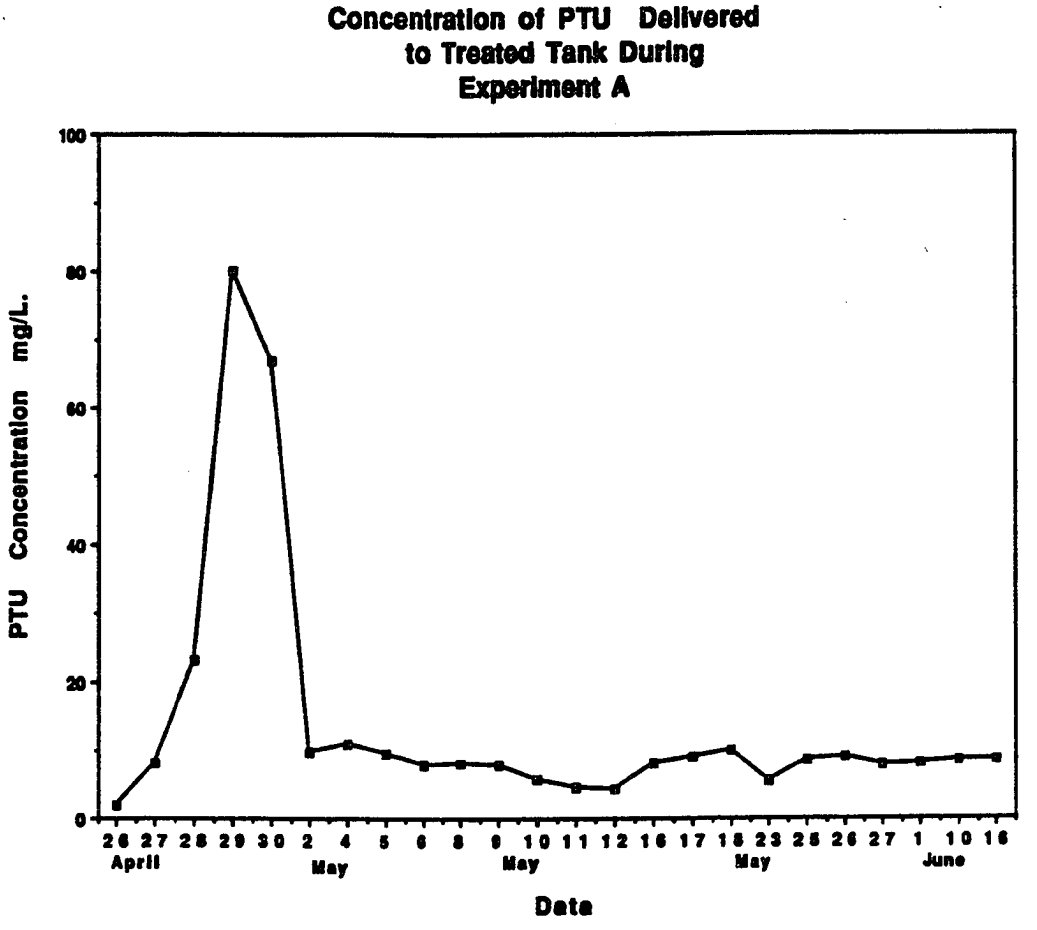

Figure A-1a. PTU concentrations during Experiment A. Concentrations were calculated from the delivery rate and concentration of PTU stock and the dilution rate by incoming water.

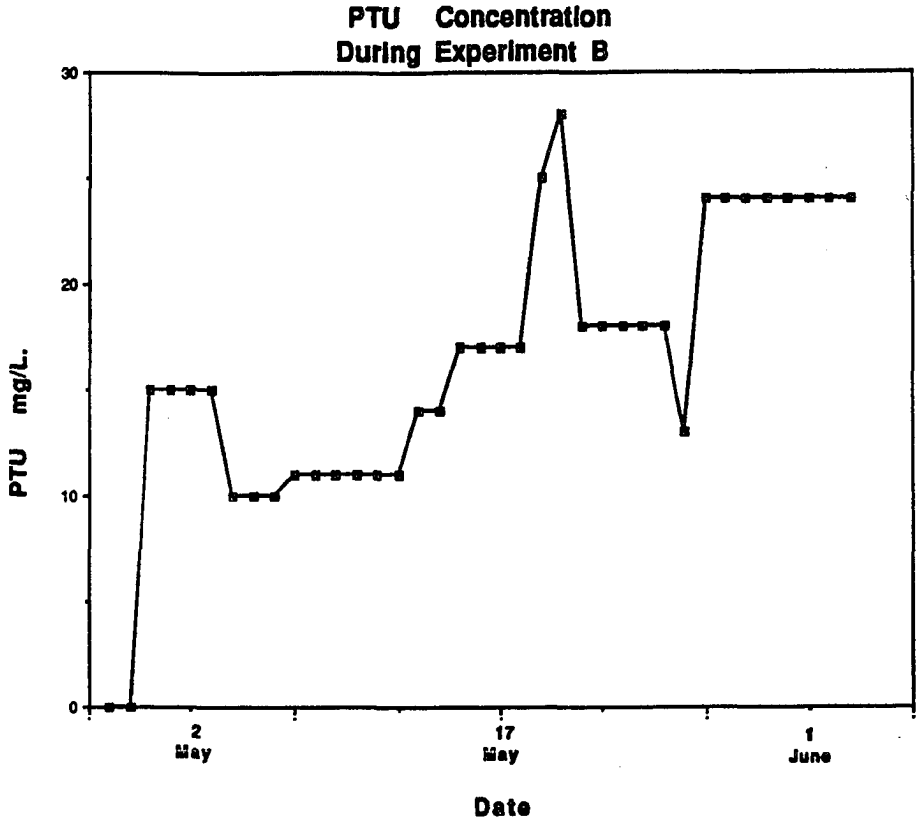

Figure A-1b. PTU concentrations during Experiment B. Concentrations were calculated as in Experiment A.

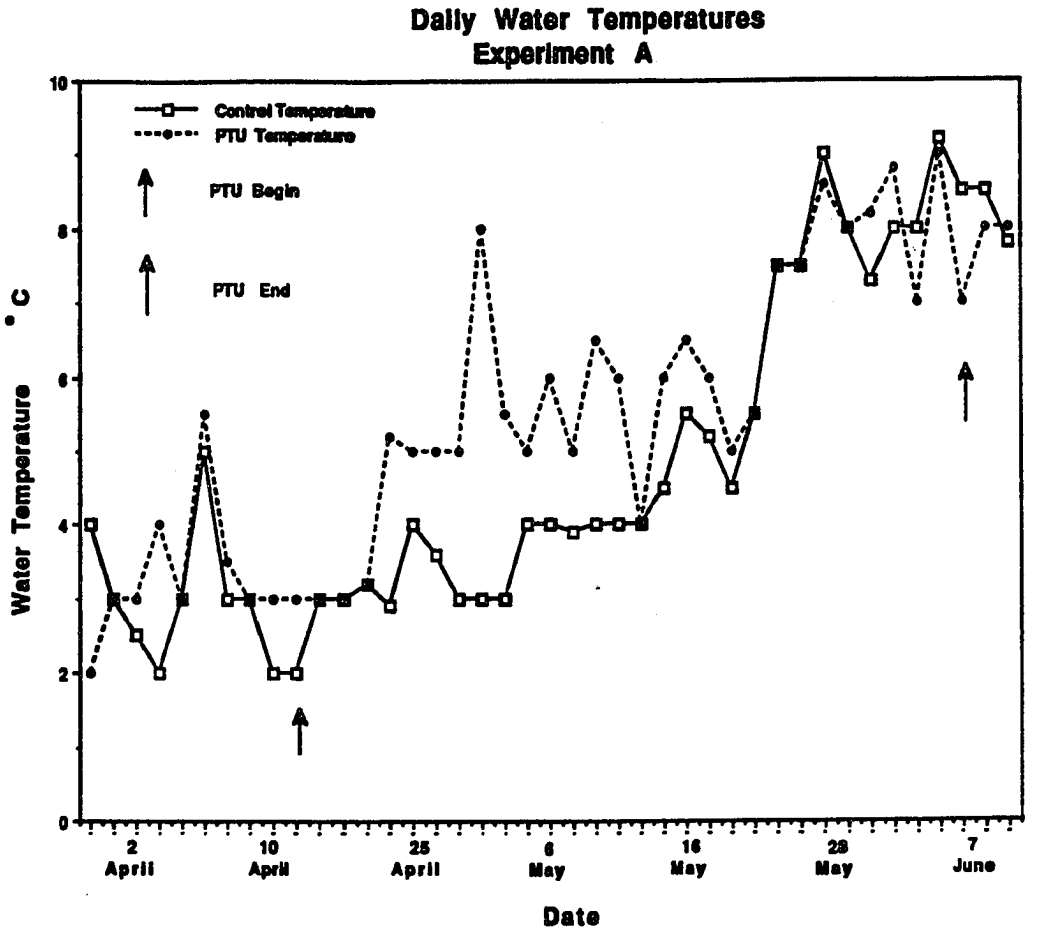

Figure A-2a. Water temperatures during Experiment A

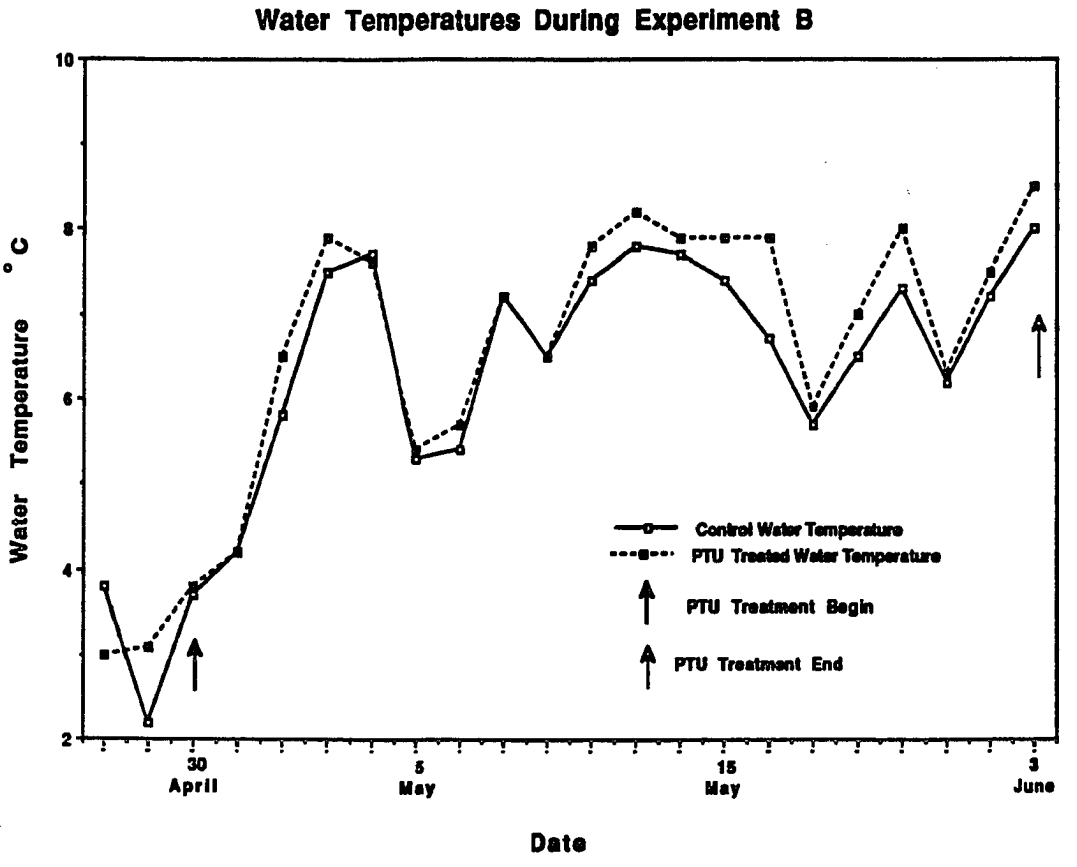

Figure A-2b. Water temperatures during Experiment B.

Figure 3a illustrates that plasma thyroxine concentrations in control fish increased rapidly during late May during a period of increasing water temperatures (Figure 2A). PTU treated fish, meanwhile, were not severely hypothyroid and at no time during the experimental period did plasma T_4 concentrations fall below a 5-10 nm baseline (3.9-7.8 ng/ml). However, T_4 plasma concentrations of PTU treated fish were significantly less than controls during the period of rapidly increasing plasma T_4 concentrations.

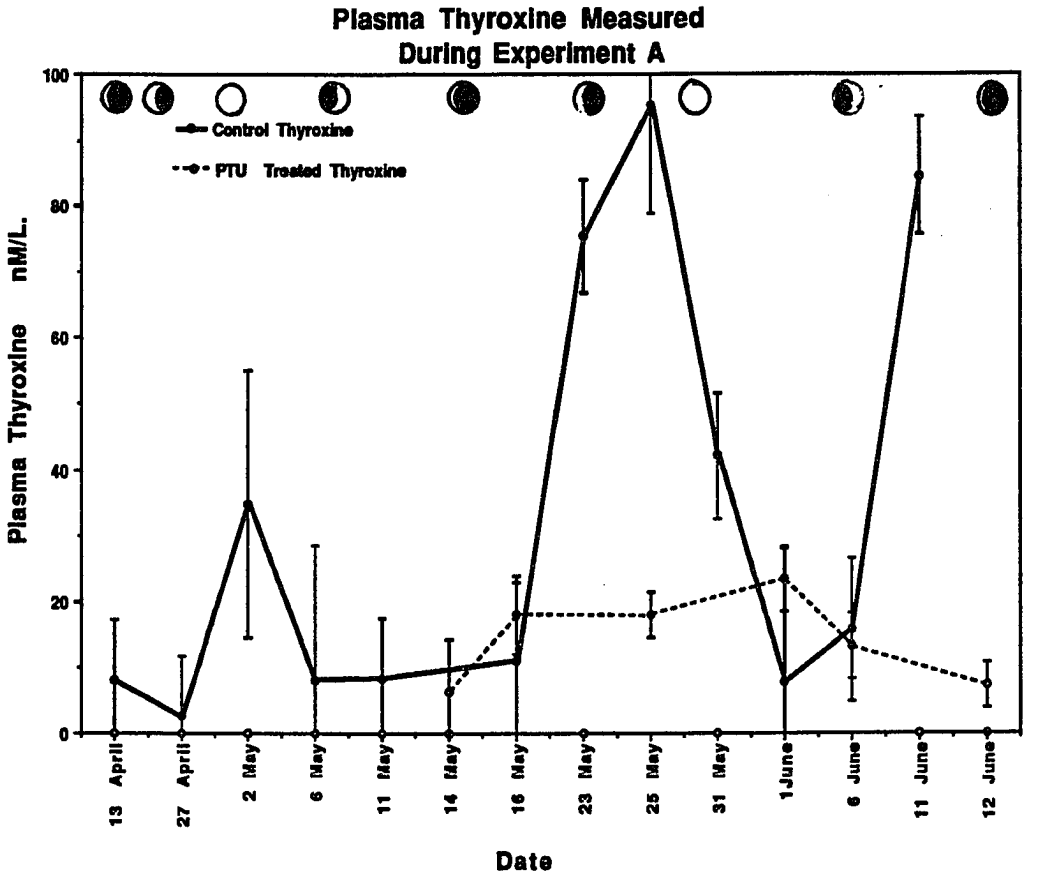

Figure A-3a. Plasma thyroxine concentrations measured during experiment A. Error bars are 95% confidence intervals. The lunar cycle is superimposed where ● = new, ◑ = first quarter, ○ = full, and ◐ = last quarter.

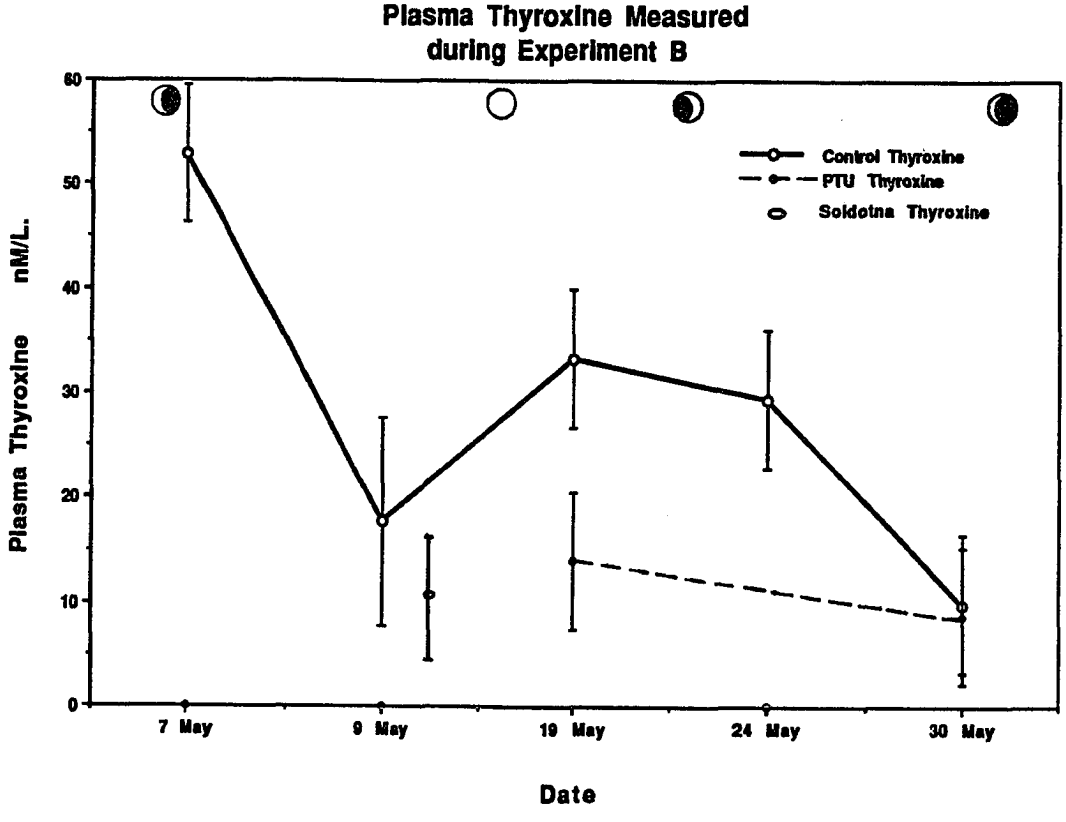

Figure A-3b. Plasma thyroxine concentrations measured during experiment B. Error bars are 95% confidence intervals. The lunar cycle is superimposed with the same symbols as in Figure A-3a.

The smolting stage was primarily determined by the degree of silvering. Subjective determinations of this gradual alternation of appearance are illustrated in Figure 4. There appears to be a correlation of smoltification stage with hormone concentration during stages 3 and 4 in the control fish; however, PTU treated fish experience a similar increase in apparent silvering (dotted line, Figure 4) and salt water tolerance (Figure 5a) with significantly reduced plasma thyroxine concentrations.

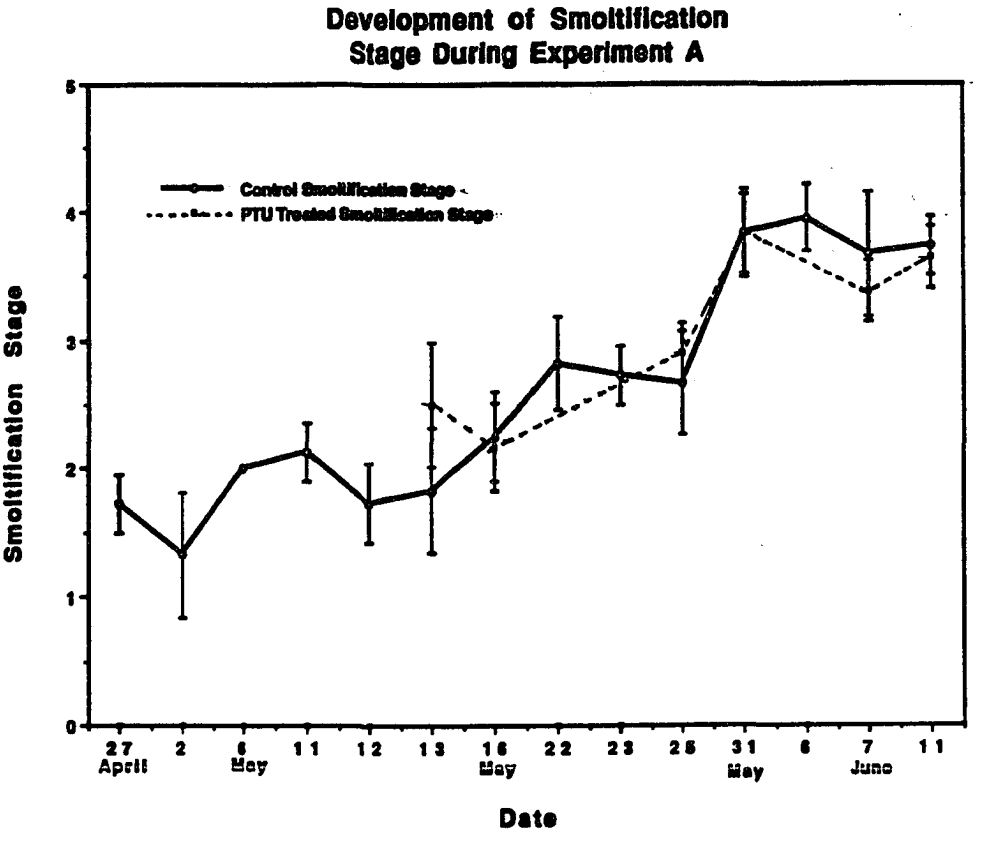

Figure A-4. Smoltification stage determined in Experiment A as described under Methods. Error bars are 95% confidence interals.

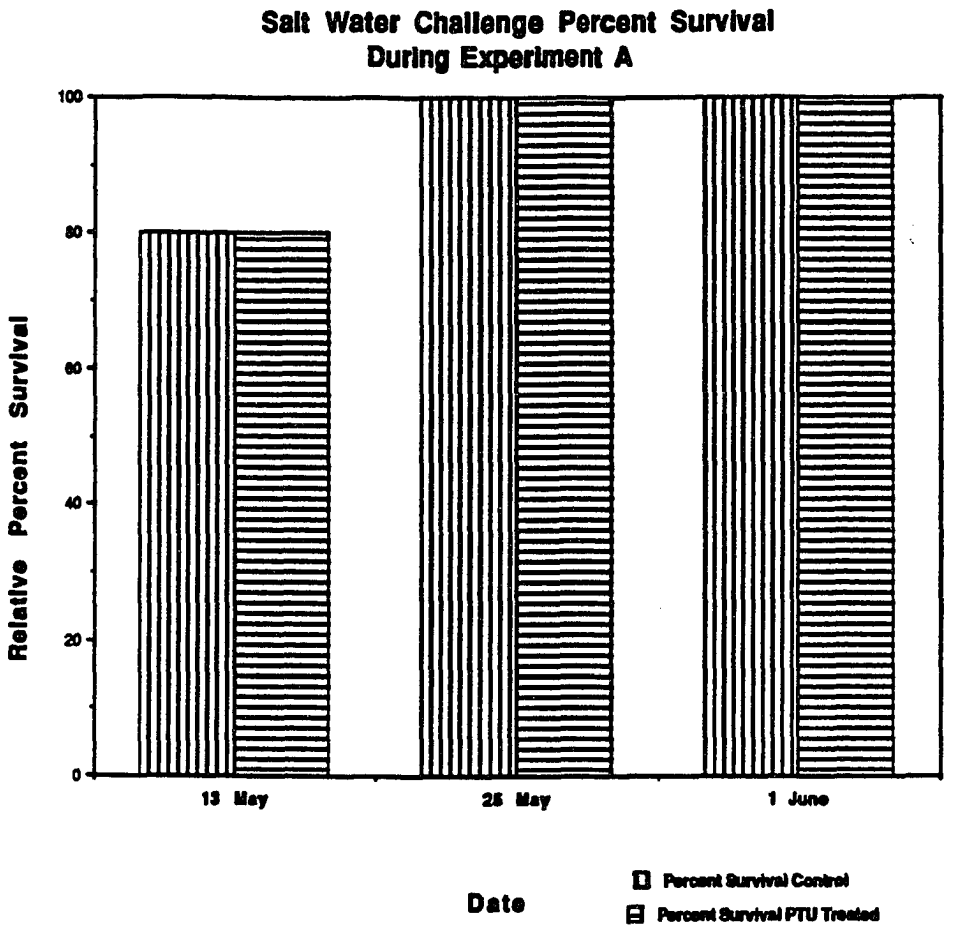

Figure A-5a. Salt water tolerance observed at selected periods during Experiment A. The bar graph represents relative percent survival following a three day exposure to full strength seawater.

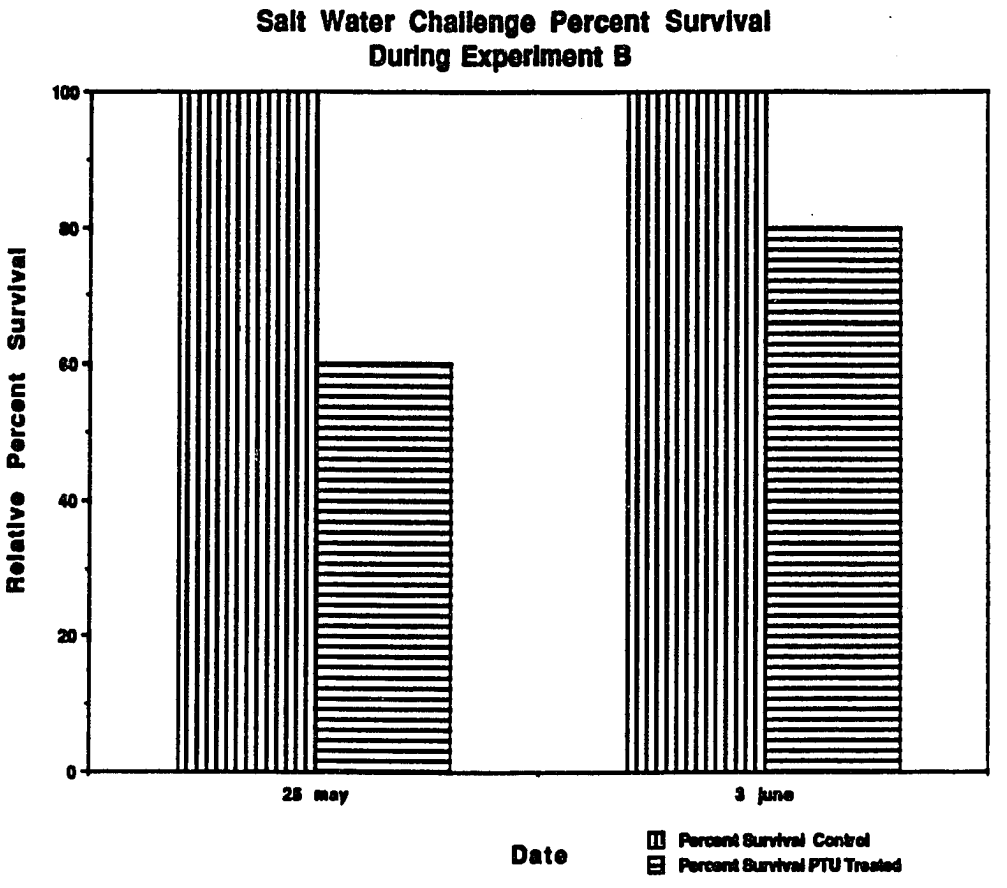

Figure A-5b. Salt water tolerance observed at selected periods during Experiment B. Percent survival is depicted as in Figure A-5a.

Both populations of fish experienced a nonsignificant decrease in condition factor after the parr marks began to fade during early smoltification (Figure 6a). Condition factors of controls and PTU treated fish are not significantly different from each other during any observation period.

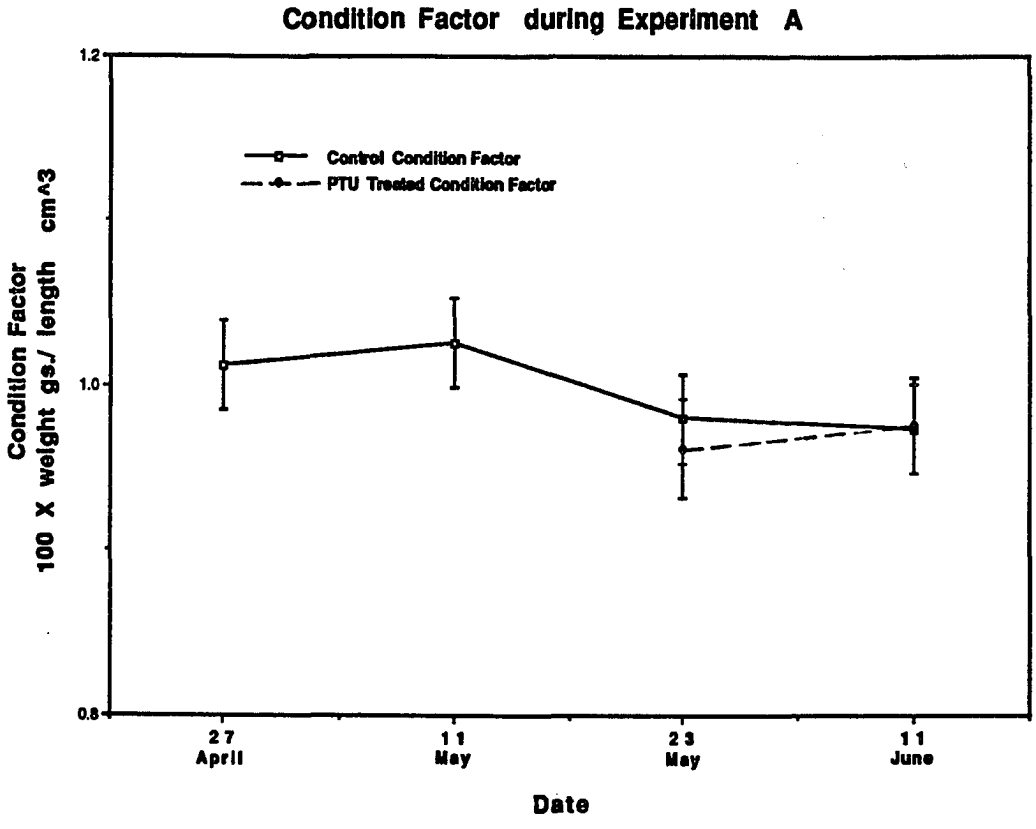

Figure A-6a. Condition factor determined as described in Methods during Experiment A. Error bars are 95% confidence intervals.

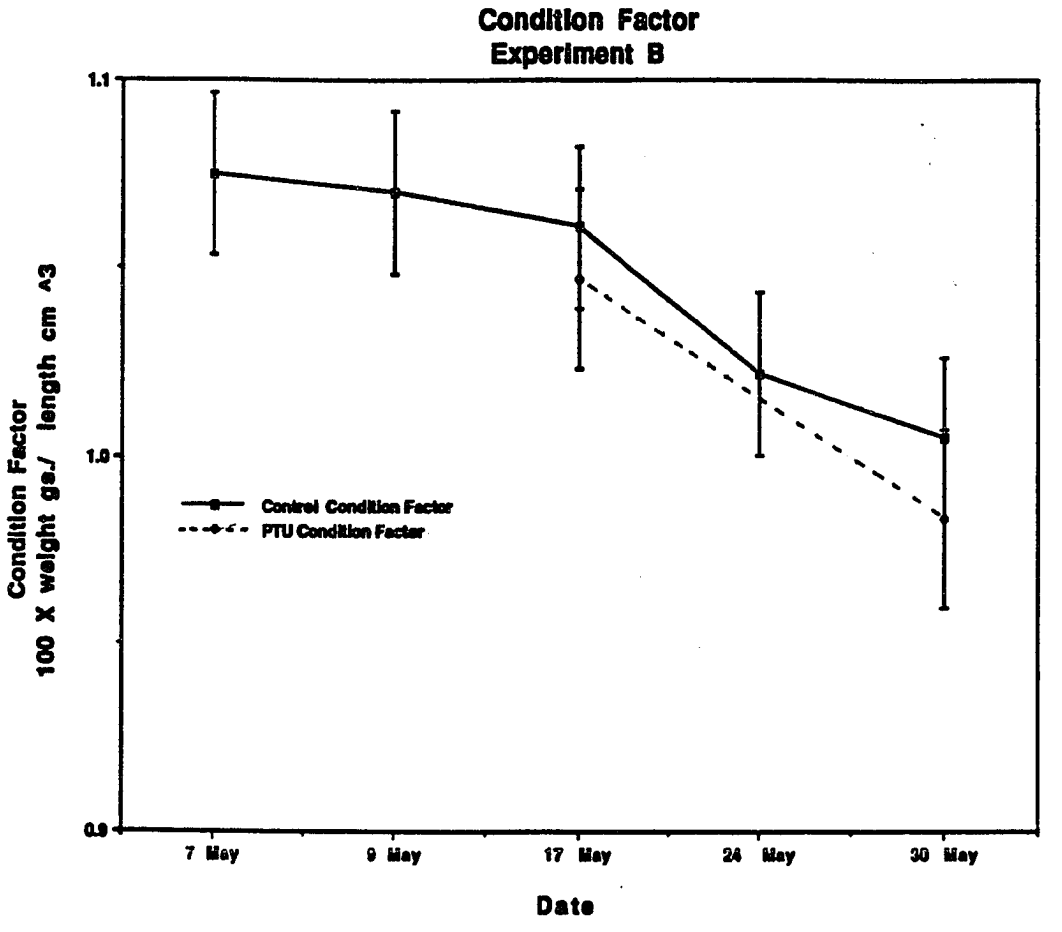

Figure A-6b. Condition factor determined during experiment B. Error bars are 95% confidence intervals

Experiment B

PTU delivery illustrated by Figure 1b began 30 April and was terminated 3 June. Goitrogen concentrations increased stepwise beginning in mid-May from 10 mg/l to 25 mg/l.

Water temperatures during Experiment B were generally warmer than the conditions of Experiment A, a reflection of the tank's exposure to sunlight, but consistent with the experimental stock's temperature history.

Figure 3b suggests that plasma thyroxine concentrations were elevated when the

fish were transfered from the Ship Creek Hatchery. Transport stress (Grau et al., 1985) may account for the significant decrease in hormone concentration over the 4 day sampling period 5-9 May. However, the lowest measured concentration was greater, but not significantly different, than hormone concentrations in presmolt Soldotna fish sampled 12 May. Plasma T_4 concentrations recovered to a plateau of 30-32 nM for the following two weeks before declining to 10 nM at the end of May. The fish maintained their smolt appearance throughout the period and, as shown in Figure 5b, successfully entered salt water when tested 25 May.

Plasma T_4 concentrations of fish reared in PTU rapidly declined to the presmolt values recorded from Soldotna fish and did not vary significantly thereafter. By the end of the experimental period, T_4 concentrations in control fish had declined such that there was no difference between control and treated fish. There did not appear to be an affect of increasing PTU load on plasma thyroxine concentrations in treated fish at the end of the experimental period.

Condition factor of control and treated fish illustrated by Figure 6b declined significantly during the experimental period. PTU treated fish were not significantly different from controls during any testing period. Salt water challenge suggested that PTU treated fish could not osmoregulate as well as controls (Figure 5b).

Lunar phases are superimposed on Figures 3a and 3b. A correlation of the serum thyroxine surge with the new moon is not apparent although a new-moon related surge may have occurred before the sampling interval began in Experiment B.

Discussion

Experiment A revealed that the elevated thyroxine concentrations observed in control fish do not appear essential for the changes in pigmentation or salt water tolerance associated with smolt transformation. Fish treated with 10 mg/l PTU did not experience the typical surge in thyroxine concentrations yet appeared to undergo smolt transformation with equal competency as controls.

During Experiment B, plasma T_4 concentrations in PTU treated fish rapidly declined to presmolt concentrations (10 nM) measured in a similar stock. While significantly different from controls, experiment B treated fish did not differ from Experiment A treated fish in spite of the increased PTU load.

Experiment B fish appear to have experienced elevated thyroxine concentrations when they were obtained from the hatchery and, according to salt water challenge records from Ship Creek Hatchery (D. Kieffer, pers. comm), were able to regulate sodium to 184 meg/l by 1 May. Therefore, the apparent decreased salt water tolerance of PTU treated fish observed at the end of May may constitute either a reversal of salt water tolerance similar to that previously reported (Baggerman, 1963) or an acceleration of parr reversion.

These experiments suggest that the surge in serum thyroxine concentrations during smoltification is attenuated by the presence of goitrogen, but in neither experiment did serum thyroxine concentrations of treated fish fall to undetectable limits. Even though PTU interferes with T_4 biosynthesis (Iino et al., 1961), PTU treated fish were not severly hypothyroid in either experiment. This could be related to the extrathyroidal effects of PTU which includes inhibition of tissue deiodination (Escobar and Del Ray, 1967; St. Germain and Croteau, 1989),

or changes in tissue binding capacity (Sullivan, 1986), either of which could affect the plasma clearance rate. Thus, in PTU treated animals the lifetime of circulating T_4 could increase and allow a reduced rate of net synthesis to maintain detectable plasma concentrations.

It appears that environmental goitrogens may affect thyroxine metabolism in smolting salmon. This observation is consistent with earlier work demonstrating that chlorinated biphenyl pollution in an iodine deficent environment results in thyroid hyperplasia and decreased serum thyroxine concentrations in Great Lakes coho salmon (Sonstegard and Leatherland, 1976). Amphibian metamorphosis can be arrested at distinct phases dependent on the sensitivity of metamorphosing systems to thyroxine concentration (Kollros, 1961). Contamination of rearing water with natural or anthrogenic goitrogens may decrease thyrogenic function during smoltification yet permit development of smolt transformation and salt water tolerance. Less obvious smoltification indices such as the change to adult hemoglobin (Sullivan et al., 1987), behavioral transformation like down-stream migration (Zaugg, et al., 1984 ; Youngson et al., 1985; Godin et al., 1974), or olfactory imprinting (Scholz et al., 1981) may be sensitive to higher thyroxine thresholds and require the large transitory increases in hormone concentration associated with smoltification. This research suggests that some smoltification indices such as salt water tolerance may be sensitive to a minimal sustained thyroxine concentration but that the episodic increase in thyroxine concentration is not necessary for the semblance of smolt transformation. In general, the data do not support the contention that the surge in serum thyroxine is "precisely timed to coincide with the new moon" (Grau et al., 1982).

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