Thyroid Activity Concomitant with Olfactory Learning and Heart Rate Changes in Atlantic Salmon, *Salmo salar*, during Smoltification[1]

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Thyroid-histological (epithelial cell height, follicular eccentricity) and thyroid-radiochemical (thyroxine, triiodothyronine) activities were examined in Atlantic salmon, *Salmo salar*, undergoing smoltification in relation to cardiac conditioning to L-cysteine (olfactory learning). Changes in plasma levels of thyroxine were also examined along with those of resting heart rate, during and after smoltification. In a related study, we reported greater learning ability in age-groups 3 (612–619 d since birth) and 6 (642–649 d) as well as a greater long-term olfactory memory in age-group 3. In the present study, thyroid-histological activity was correlated with olfactory learning during smoltification. Higher histological values occurred concomitantly with greater learning in age-groups 3 and 6. During smoltification, changes in thyroid-histological activity were different from those of radiochemical activity. Apparently different plasma–tissue fluxes of thyroid hormones occurred between age-groups 3 and 6. Plasma thyroxine was correlated with resting heart rate. Our results suggested that thyroid hormones play a role in olfactory learning and imprinting in anadromous salmonids.

Les caractéristiques histologiques (hauteur des cellules épithéliales, excentricité folliculaire) et radiochimiques (thyroxine, triiodothyronine) thyroidiennes sont examinées chez des saumons atlantiques, *Salmo salar*, au cours de la "smoltification" en relation avec le conditionnement d'une réponse cardiaque à la L-cystéine (apprentissage olfactif). Les changements des niveaux plasmatiques de thyroxine sont également évalués avec ceux de la fréquence cardiaque au repos, pendant et après la smoltification. Parallèlement, nous avons décelé une plus grande habileté à l'apprentissage olfactif chez les groupes d'âge 3 (612–619 d depuis la naissance) et 6 (642–649 d) ainsi qu'une meilleure mémoire olfactive à long terme chez le groupe d'âge 3. Dans le cadre de la présente étude, l'activité histologique thyroidienne est corrélée avec l'apprentissage olfactif pendant la smoltification. Des valeurs histologiques plus élevées sont apparues concomitamment à un plus grand apprentissage olfactif chez les groupes d'âge 3 et 6. Au cours de la smoltification, les changements dans l'activité histologique thyroidienne étaient différents de ceux de l'activité radiochimique. Des flux plasma-tissulaires apparemment différents d'hormones thyroidiennes se sont produits entre les groupes d'âge 3 et 6. La thyroxine plasmatique était corrélée avec la fréquence cardiaque au repos. Nos résultats suggèrent que les hormones thyroidiennes jouent un rôle dans l'apprentissage et dans l'empreinte olfactive chez les salmonidés anadromes.

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In salmonids, an active thyroid gland at the time of smoltification (Hoar 1939; Dickhoff et al. 1978) or during seaward migration (Hoar and Bell 1950; Hoar 1951) has been associated with hyperactivity (Fontaine et al. 1952; Leloup and Fontaine 1960) and also with a heightened sensitivity (or excitability) to external stimuli (Hoar 1953; Baggerman 1960; Saunders and Henderson 1970; Fontaine 1975). Salmonid juveniles injected with thyroid hormones become more active (Hoar et al. 1952; Hoar et al. 1955), more sensitive to odors (reviewed by Gorbman 1969), and better in learning ability (Lindberg and Scholz, cited in Scholz 1980). A neuronal basis for these effects is indicated by the recent findings of an intranuclear triiodothyronine (T3) binding site in the brain of salmonids exhibiting several characteristics of mammalian T3 receptors (Scholz et al. 1985; Bres and Eales 1988). Besides regulating the morphological, physiological, and behavioral changes of the parr–smolt transition (reviewed by Hoar 1976), these observations suggest that thyroid hormones affect the central nervous system of salmonid juveniles during smoltification.

The influence of thyroid hormones on growth, development, and normal functioning of the central nervous system is best known in higher vertebrates (Ford and Cramer 1977; Nunez 1984). There is a critical period in neonatal mammals during which thyroid hormones play an essential role in differentiating brain-cell structures (Hamburgh 1969). Hypothyroidism during this period causes permanent alterations of normal brain functioning characterized by behavioral defects including learning

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impairments (Meisami 1983; Tamasy et al. 1986; Rial et al. 1987). Relatively high concentrations of brain nuclear T3 receptors (Oppenheimer et al. 1974; Gullo et al. 1987; Ferreiro et al. 1988) have been identified, suggesting the existence of a thyroid–brain mechanism regulating the functioning of the nervous system during development.

The precision of homing behavior in anadromous salmonids has been attributed to the existence of a sensitive period for olfactory imprinting occurring during smoltification (reviewed by Hasler and Scholz 1983). Scholz (1980) hypothesized that high levels of thyroid hormones facilitate olfactory imprinting in coho salmon, *Oncorhynchus kisutch*, undergoing smoltification. Hasler and Scholz (1983) reported an experiment in which presmolt cohos injected either with gonadotropin (ACTH) to induce a migratory state or with a saline solvent (SS) were divided in two subgroups and further injected with the thyroid stimulating hormone (TSH) or with SS concomitantly with exposure to an artificial odorous chemical (morpholine or phenethyl alcohol). All fish were subsequently tested in a stream scented with the artificial odorous chemical. The results of this experiment showed that fish treated with ACTH + TSH as compared with those injected differently (ACTH + SS, SS + TSH, or SS + SS) were attracted more reliably to their imprinted odor. Heart rate responses to imprinting odors were also examined in these fish. Again, compared with any other group of injected fish, those treated with ACTH + TSH reacted significantly more to the imprinted odor by an unconditioned cardiac deceleration, as when they were stimulated with a nonfamiliar chemical. Hasler and Scholz (1983) concluded that TSH injections activated olfactory imprinting in smolts. Through their effect on the central nervous system, thyroid hormones may influence the imprinting process. Newly hatched birds imprint until a few hours after birth (Hess 1973) during which high levels of thyroid hormones are found in their blood. (Thommes et al. 1977). Delayed maturation of the central nervous system of prehatched birds by thyroid-blocking agents (Chandrasekhar et al. 1979) may interfere with the imprinting process.

A sensitive period for olfactory imprinting (SPOI) has been identified in Atlantic salmon, *Salmo salar*, undergoing smoltification (Morin et al. 1989). The magnitude of a conditioned cardiac response to L-cysteine was measured in fish during six 8-d intervals within the parr–smolt transition. We postulated that odor learning would be optimal during a sensitive period for imprinting and that the magnitude of a conditioned cardiac deceleration would significantly increase at such times. In addition, olfactory memory was assessed by exposing fish to L-cysteine during the same six 8-d intervals of smoltification and by measuring their unconditioned cardiac deceleration to L-cysteine after smoltification. We postulated that the magnitude of cardiac deceleration to L-cysteine would be significantly greater among fish exposed to L-cysteine during a sensitive period for imprinting. Greater conditioned cardiac decelerations to L-cysteine occurred in age-groups 3 and 6 as compared with any other age group of smoltification. Fish tested for odor recognition exhibited a greater unconditioned cardiac deceleration to L-cysteine if they had been exposed during the third age interval of smoltification. Morin et al. (1989) concluded that there are two distinct optimal intervals for olfactory learning (age-groups 3 and 6) during smoltification but that there is only one "sensitive" period for olfactory imprinting (age-group 3), occurring between 21 and 28 d after the onset of smoltification induced in the laboratory.

The principal objective of the present study was to determine in Atlantic salmon if a relationship exists between thyroid hormones, olfactory learning, and olfactory imprinting. We proposed that higher thyroid activity significantly correlated with greater ability for heart rate conditioning to L-cysteine during smoltification would be evidence of the role of thyroid hormones in olfactory learning (age-groups 3 and 6) or in olfactory imprinting (age-group 3).

To assess thyroid activity during smoltification, we used two histological measures, epithelial cell height and follicular eccentricity, and two radiochemical measures, plasma levels of thyroxine (T4) and T3. Epithelial cell height generally increases with plasma levels of thyroid hormones during smoltification, indicating a heightened thyroid activity (Eales 1963, 1965; Nagahama et al. 1982). When thyroid activity is least, follicular cells are generally flattened, but when greater, the cells are cuboid (Hibiya 1982) or columnar (Barrington 1975; Bloom and Fawcett 1975). Infolding and tufting follicles have also been identified in very active glands of fish during smoltification (Hoar 1939; Fontaine et al. 1952), suggesting that the shape of thyroid follicles changes with thyroid activity. Since we found, in a preliminary study, that the tendency of circular follicles to become elliptic increases with cell height during smoltification, we quantified this tendency with a measure of "follicular eccentricity" as an additional and novel index of thyroid activity during smoltification.

Another objective was to assess the relationship between resting heart rate and plasma levels of T4 during and after smoltification. Thyroid hormones are known to increase heart rate, presumably by affecting the excitability of the nervous system (Guyton 1986). Injections of small quantities of T4 in the blood of rainbow trout, *Salmo gairdneri*, increase heart rate and the excitability of the nervous system to chemical stimuli (Oshima et al. 1972). We proposed that high levels of T4 significantly correlated with high values of resting heart rate during smoltification would be evidence of the role of thyroid hormones on the excitability of fishes' nervous system.

Materials and Methods

Fish and Overall Procedure during and after Smoltification

The two hundred and forty 2-yr-old Atlantic salmon were those described in Morin et al. (1989). One hundred and twenty fish had been trained during smoltification for heart rate conditioning to L-cysteine or with a random odor–shock presentation (Experiment 1, Morin et al. 1989) whereas another 120 fish had been preexposed to L-cysteine during smoltification and tested for odor recognition after smoltification (Experiments 2 and 3, Morin et al. 1989). In the present study, we assessed (1) thyroid-histological and thyroid-radiochemical activities in fish conditioned to L-cysteine during smoltification and (2) plasma levels of T4 and resting heart rate in fish conditioned to L-cysteine or trained with a random odor–shock presentation during smoltification and in those preexposed to L-cysteine during smoltification and tested for odor recognition after smoltification. The age since birth of conditioned fish from each of the six age groups of smoltification (age-groups 1–6) was as follows: age-group 1: 592–599 d, age-group 2: 602–609 d, age-group 3: 612–619 d, age-group 4: 622–629 d, age-group 5: 632–639 d, age-group 6: 642–649 d.

When tested for odor recognition during the postsmoltification period, the age of each group of fish (age-

groups 7–12) preexposed during six equal intervals within smoltification (age-groups 1–6) was as follows: age-group 7: 654 d, age-group 8: 664 d, age-group 9: 674 d, age-group 10: 684 d, age-group 11: 694 d, age-group 12: 704 d.

We used an additional 16 fish in this study (see age-group 11 in Fig. 1) which had already been trained for heart rate conditioning to L-cysteine in one of our pilot studies. All other details concerning subjects, apparatus, and the procedure used for cardiac conditioning to L-cysteine are found in Morin et al. (1989).

Thyroid Histology

Fish were sacrificed at times between 1000 and 1300 the day after conditioning. Sampling to collect thyroid tissues occurred at 2-d intervals between 1 January and 26 February 1986. Glandular histology was performed on each fish used for Experiment 1 (Morin et al. 1989). Following plasma sampling (see below), the subpharyngeal tissue containing the thyroid gland was dissected out and fixed in a Bouin solution for light microscopy. Each piece of tissue was embedded in paraffin and 5- to 6-μm-thick serial sections were made. Samples were stained with hematoxylin–eosin or with periodic acid shift. Thyroid structures were magnified, photographed, and measured on an electronic plotting table connected to an IBM microcomputer.

Thyroid activity was assessed by measuring the height of follicular epithelial cells and follicular eccentricity (i.e. deviation from an elliptic shape). All follicles in a given fish were measured and an average size was estimated. For each fish, five follicles measuring within 1 standard deviation of the average follicle size were randomly selected. They were measured for their eccentricity and the thickness of their epithelial lining (epithelial cell height). Eccentricity (E) was derived from the following calculation:

$$E = \sqrt{1 - \left(\frac{a}{b}\right)^2}$$

where a = smallest diameter and b = largest diameter.

Plasma Collection and Thyroid Hormone Determination

Sampling to collect plasma occurred at 2-d intervals between 1 January and 26 February 1986 and at 10-d intervals between 3 March and 22 April of the same year. After fish had been lightly anesthetized and assessed for body coloration, the tail (wiped dry) was severed and blood was aspirated from the caudal section of the dorsal aorta into heparinized tuberculin syringes. The blood was transferred to microhematocrit capillary pipettes treated with heparin for centrifugation. Upon separation from blood, the plasma was stored frozen at −20°C in 0.3-mL plastic microcentrifuge tubes until assayed for T4 and T3.

The quantity of blood taken from each fish was sufficient for T4 determination but not for T3. Consequently, a T3-radiochemical analysis was performed for each age group of smoltification on a single "pooled" sample containing the blood of all 20 fish trained in the presence of L-cysteine (see Experiment 1 in Morin et al. 1989). Hormone determinations on pooled samples (T3) or on individual samples (T4) were made with radioimmunoassay test kits (T4: Becton Dickinson Co.; T3: Kallestad Laboratories, Inc.). Since these tests are clinically used for human thyroid hormones, aliquots of the standards had to be made for plotting a standard curve fit to identify small quantities of hormones typically found in salmon plasma. Standards and samples were run in duplicate. Calculation of radioimmunoassay results was made by a LKB-Wallac Rack-gamma counter utilizing the spline-function program.

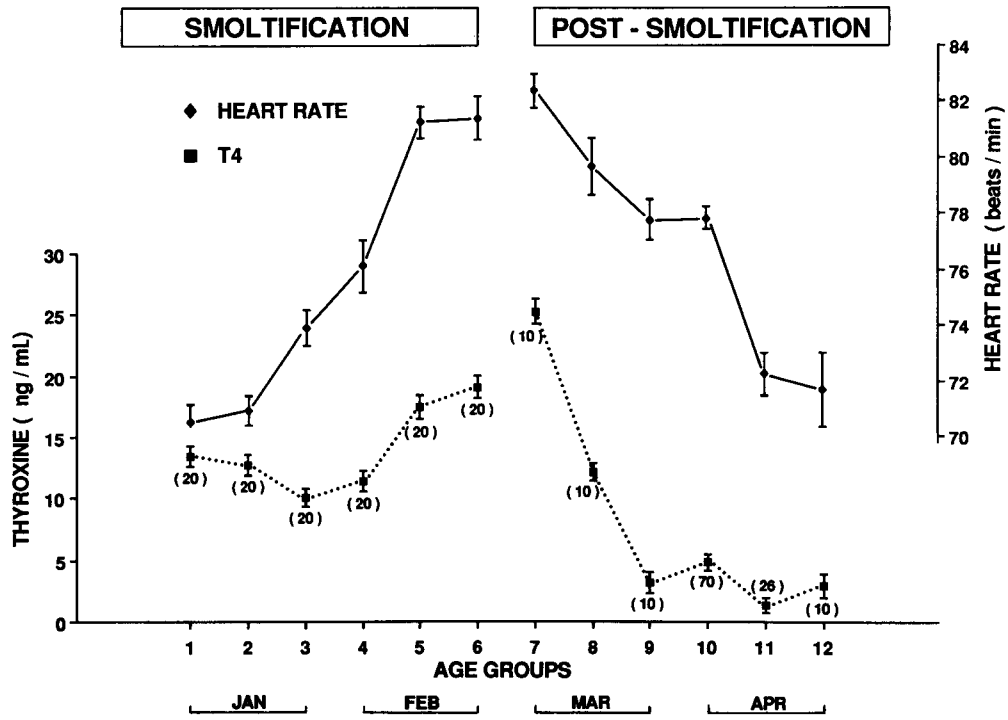

FIG. 1. Physiological changes in 2-yr-old Atlantic salmon between the beginning of January and the end of April 1986. Heart rate and plasma thyroxine (T4) levels were measured in six age groups of smoltification and six age groups of postsmoltification. Size of samples is shown in parentheses and the vertical bars represent the mean ± SE.

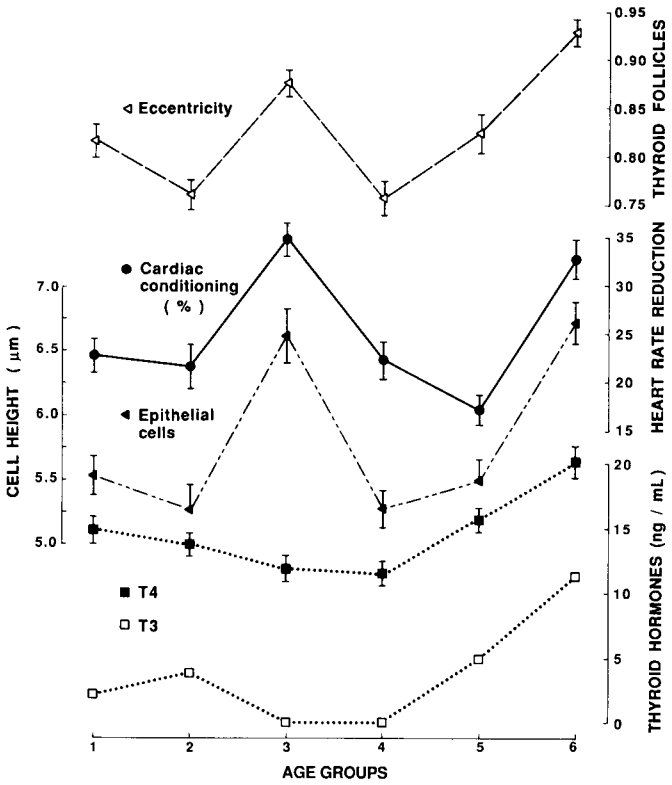

FIG. 2. Relationships between heart rate conditioning to L-cysteine and thyroid activity during smoltification, in 2-yr-old Atlantic salmon. Cardiac conditioning was measured at the end of the second testing session (day 2). Plasma levels of thyroxine (T4) and triiodothyronine (T3), epithelial cell height, and follicular eccentricity measurements were derived from samples collected on day 3. An eccentricity score of 1 is indicative of a perfect ellipse, while a score of 0 describes a circle. The vertical bars represent the mean ± SE.

Results

T4 and Resting Heart Rate during and after Smoltification

From a simple regression analysis, the pattern of variation in resting heart rate from age-group 1 to 12 was similar to that of plasma levels of T4 ($F = 17.06$; df = 1,118; $P < 0.01$; Fig. 1). Consistent differences occurred from age-group 1 to 12 in fish's heart rate ($F = 7.85$; df = 11,244; $P < 0.01$; one-way ANOVA) as well as in plasma levels of T4 ($F = 59.39$; df = 11,244; $P < 0.01$). Heart rate increased rapidly from age-group 1 to 5, stabilized at age-group 6 and 7, and then decreased regularly until the end of the study (age-group 12). Levels of T4 remained unchanged from age-group 1 to 4 but then increased regularly to reach a maximum of 25.2 ng/mL for age-group 7. A drastic reduction in T4 followed for age-group 8, and from age-group 9 to 12, small quantities of T4 remained in fishes' blood.

Olfactory Learning and Thyroid Activity during Smoltification

Figure 2 depicts thyroid activity and heart rate reduction in different age groups of 10 fish conditioned to L-cysteine during smoltification. Data from fish trained under a random pairing situation (see Morin et al. 1989) are not included here. It is worth mentioning, however, that for each index of thyroid activity analyzed separately (follicular eccentricity, epithelial cell height, or plasma levels of T4), there was no difference between the values obtained in conditioned fish and those obtained in the random pairing group (t-tests). This should be kept in mind considering that each mean value of plasma T4 shown in Fig. 1 and calculated from 20 fish (conditioning and random pairing groups) looks somewhat different from that shown in Fig. 2, calculated from only 10 conditioned fish.

From simple regression analyses, changes in the magnitude of cardiac conditioning, across the different age groups of smoltification, were similar to those of epithelial cell height ($F = 5.60$; df = 1,58; $P < 0.05$) and of follicular eccentricity ($F = 4.07$; df = 1,58; $P < 0.05$). In addition, changes in epithelial cell height were similar to those of follicular eccentricity ($F = 4.28$; df = 1,58; $P < 0.05$). Changes in plasma T3 were not analyzed statistically due to sample size. Nevertheless, from age-group 2 to 6, changes in plasma T3 resembled those of plasma T4. Plasma T4 was not correlated with cardiac conditioning, epithelial cell height, or follicular eccentricity.

Overall differences between the age groups of smoltification were found for cardiac conditioning ($F = 4.98$; df = 5,54; $P < 0.01$; one-way ANOVA), epithelial cell height ($F = 4.79$; df = 5,54; $P < 0.01$), and follicular eccentricity ($F = 6.45$; df = 5,54; $P < 0.01$). For cardiac conditioning, cell height, and follicular eccentricity, significant differences ($P < 0.01$) between successive age groups existed between age-groups 2 and 3, 3 and 4, as well as between age-groups 5 and 6 (Duncan's multiple range test). From age-group 1 to 6, overall differences in plasma T4 levels were found in conditioned fish ($F = 6.02$; df = 5,54; $P < 0.01$). Plasma T4 remained unchanged from age-group 1 to 4 and then increased significantly from age-group 4–5 and from age-group 5–6 ($P < 0.05$).

Discussion

Plasma levels of T4 increased during smoltification to subsequently decline during the postsmoltification period. This pattern of T4 fluctuations is consistent with previous studies on Atlantic salmon undergoing smoltification under natural (Lindahl et al. 1983; Boeuf and Prunet 1985; Dickhoff et al. 1985; Virtanen and Soivio 1985; McCormick et al. 1987) or long but constant photoperiods (Virtanen and Soivio 1985; McCormick et al. 1987). Despite the high functional state of the thyroid in fish from age-group 3, it can be argued that thyroid activity was relatively low in those fish, since the "surge" of plasma T4 was most apparent near the end (age-groups 5 and 6) and soon after smoltification (age-group 7). However, compared with recent studies on Atlantic salmon reporting concentrations of plasma T4 of about 10 ng/mL at the onset of smoltification (Boeuf and Prunet 1985; Dickhoff et al. 1985; Virtanen and Soivio 1985; McCormick et al. 1987), our initial value of T4 measured in fish from age-group 1 of smoltification was relatively high, as it reached about 14 ng/mL.

A key issue of this research was to show significant positive correlations between thyroid-histological activity and olfactory learning in Atlantic salmon undergoing smoltification. More specifically, we have shown that a greater ability for cardiac conditioning to L-cysteine, as in fish from age-groups 3 and 6, was associated with higher values of epithelial cell height and follicular eccentricity. This suggests that thyroid hormones facilitate olfactory learning during specific intervals within smoltification. Thyroid hormones may facilitate olfactory learning by affecting the excitability of fishes' nervous system. In higher vertebrates, these hormones are known to affect excitability by increasing the activity of adrenergic receptors (Had-

ley 1988). As in some other teleosts, the heart of salmonids possesses adrenergic receptors (Laurent et al. 1983) whose sensitivity may be altered by thyroid hormones. Our results showing a significant correlation between plasma levels of T4 and resting heart rate indicate that thyroid hormones influence heart rate by affecting the excitability of fishes' nervous system. This is consistent with the observations of Oshima et al. (1972) who reported lower heart rate and lower excitability to chemical stimuli (EEG recordings) in thyroidectomized as compared with normal rainbow trout. They also reported higher heart rate and higher excitability to chemical stimuli in thyroidectomized as well as in normal fish injected with a low dosage of T4.

A discrepancy between histological and radiochemical indexes of thyroid activity existed in fish from age-group 3 but not in fish from age-group 6. In fish from age-group 3, a significant increase in epithelial cell height and follicular eccentricity occurred in the absence of any change in plasma T4. In contrast, a significant increase in cell height and follicular eccentricity occurred in fish from age-group 6 concomitantly with a significant increase in plasma T4. Such differences in T4 kinetics between fish from age-groups 3 and 6 coincide with contrastingly different phenomena: olfactory learning (age-groups 3 and 6) and olfactory imprinting (age-group 3). Morin et al. (1989) reported a significantly greater ability for olfactory learning in fish from age-groups 3 and 6 but a significantly greater capacity for long-term olfactory memory only in fish from age-group 3. These findings suggest that thyroid hormones may influence olfactory imprinting by exerting a specific thyroid–brain effect rather than a general effect on the fishes' nervous system. This hypothesis is consistent with the idea of a thyroid–brain mechanism activating olfactory imprinting in anadromous salmonids (Hasler and Scholz 1983; Scholz et al. 1985).

The discrepancy between plasma T4 and any of the thyroid-histological parameters, observed in smolts preexposed to L-cysteine as age-group 3, may be due to an incorporation of thyroid hormones by the brain and other target organs. We speculate that a rapid plasma–tissue flux of T4 occurred in fish from age-group 3 preventing a plasma T4 surge following heightened thyroid activity. Since morphological changes accompanying smoltification persisted in fish from age-group 3 (Morin et al. 1989), body tissues probably required great quantities of thyroid hormones to process these changes. This interpretation is supported by the findings of Specker et al. (1984) showing significantly greater quantities of T4 in tissues than in plasma of coho salmon during smoltification. At the onset of this developmental process, an efficient plasma–tissue flux of T4, lowering the level of T4 in fishes' blood, may indicate the involvement of slowly equilibrating tissues (Specker et al. 1984) including skin and brain as major targets of T4. Evidence that rapid incorporation of thyroid hormones by the brain (and other tissues) lowers plasmatic levels of these hormones was provided by Scholz et al. (1985) who found a progressive decline in blood T3 together with a gradual uptake of T3 in brain nuclei as well as in the whole brain tissue of rainbow trout injected with a tracer dose of radiolabelled T3.

Discrepancies between histological (i.e. cell height) and radiochemical indexes are reported elsewhere (Fontaine et al. 1953; Eales 1963, 1964, 1965; Drury and Eales 1968; Nishioka et al. 1985). Lack of correlation between these different indexes has been attributed to the heterogeneity of fishes' thyroid characterized by the coexistence within the same tissue of follicular units in different functional states (Eales 1979). Previous histological examinations have been unreliable (Sage and Robins 1970) and have based their measurements on individual epithelial cells from follicles selected at random on a given fish (Higgs et al. 1976; Milne and Leatherland 1980). To increase reliability in our assessment of thyroid activity, we took two instead of only one histological index. In addition, we measured the height of all epithelial cells in representative thyroid follicles. Furthermore, follicular eccentricity is a good sign of thyroid activity, as it was significantly correlated with epithelial cell height, a common criterion of thyroid activity. Higher follicular eccentricity in fish from age-group 3 or 6 could have been the result of follicle proliferation in highly active thyroids. This might have led follicles growing close to each other within the restricted confines of the basibranchial region to become mechanically distorted due to a "packing effect" (J. G. Eales, University of Manitoba, Winnipeg, Man., pers. comm.).

Greater olfactory learning reflecting an optimal capacity for encoding odor-related information during a smoltification-associated SPOI, together with long-term retention of odor information acquired during this sensitive period, may be attributed to the influence of thyroid hormones on brain maturation (Scholz and Hasler 1983; Scholz et al. 1985). Other suggestions have been made that imprinting occurs under high arousal (Kovach 1970; Martin and Schutz 1975) and high excitability (Bateson 1981) with important structural alterations in the central nervous system (Horn 1986). Internal changes involving hormones may also increase sensitivity to environmental stimuli at the time of imprinting in vertebrates (Landsberg and Weiss 1976; Weiss et al. 1977) including anadromous salmonids (Fontaine 1975).

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