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Evidence that thyroid hormone induces olfactory cellular proliferation in salmon during a sensitive period for imprinting

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Summary

Salmon have long been known to imprint and home to natal stream odors, yet the mechanisms driving olfactory imprinting remain obscure. The timing of imprinting is associated with elevations in plasma thyroid hormone levels, with possible effects on growth and proliferation of the peripheral olfactory system. Here, we begin to test this idea by determining whether experimentally elevated plasma levels of 3,5,3′-triiodothyronine (T_3) influence cell proliferation as detected by the 5-bromo-2′-deoxyuridine (BrdU) cell birth-dating technique in the olfactory epithelium of juvenile coho salmon (*Oncorhynchus kisutch*). We also explore how natural fluctuations in thyroxine (T_4) relate to proliferation in the epithelium during the parr–smolt transformation. In both studies, we found that BrdU labeled both single and clusters of mitotic cells. The total number of BrdU-labeled cells in the olfactory epithelium was significantly greater in fish with artificially elevated T_3 compared with placebo controls. This difference in proliferation was restricted to the basal region of the olfactory epithelium, where multipotent progenitor cells differentiate into olfactory receptor neurons. The distributions of mitotic cluster sizes differed significantly from a Poisson distribution for both T_3 and placebo treatments, suggesting that proliferation tends to be non-random. Over the course of the parr–smolt transformation, changes in the density of BrdU cells showed a positive relationship with natural fluctuations in plasma T_4. This relationship suggests that even small changes in thyroid activity can stimulate the proliferation of neural progenitor cells in the salmon epithelium. Taken together, our results establish a link between the thyroid hormone axis and measurable anatomical changes in the peripheral olfactory system.

Key words: thyroid hormone, olfactory imprinting, cell proliferation, neural progenitor cell, peripheral olfactory system, parr–smolt transformation, salmon, *Oncorhynchus kisutch*.

Introduction

Salmon are well known for their dramatic and accurate homing migrations guided by an imprinted olfactory memory of their natal stream. The sensitive period for olfactory imprinting is linked to a specific period of development – the parr–smolt transformation (for reviews, see Hasler and Scholz, 1983; Dittman and Quinn, 1996). The parr–smolt transformation, also known as ‘smolting’, is a transitional period where individuals undergo a suite of morphological, physiological and behavioral changes that prepare the stream-dwelling parr for life in seawater (for review, see McCormick et al., 1998). Some of these changes are driven by the thyroid hormone thyroxine (T_4; Dickhoff et al., 1982; Yamauchi et al., 1984; Dickhoff and Sullivan, 1987).

Hasler and Scholz (1983) suggested that the sensitive period for olfactory imprinting in salmon is tied to changes in T_4 that occur during smolting. In a now classic study, they showed that hatchery-reared coho salmon (*Oncorhynchus kisutch*) learned, remembered and homed to synthetic odors if they were exposed to them during the parr–smolt transformation (Scholz et al., 1976). In a similar study, Dittman et al. (1996) exposed hatchery-reared coho salmon to synthetic or natural stream odors at several stages of development and found that only fish that were exposed to odors during smolting formed an imprinted memory. In addition, artificially elevating thyroid hormone induced parr to imprint to artificial odorants, while parr with unaltered hormone levels did not (Scholz, 1980).

We have suggested that the relationship between thyroid hormone and olfactory imprinting may involve differential growth of the peripheral olfactory system (e.g. Nevitt and Dittman, 2004). The olfactory nerve and glomerular structures in the bulb grow dramatically during smolting in both coho and Atlantic salmon (*Salmo salar*; Jarrard, 1997), and changes in the physiological sensitivity of olfactory receptor neurons to imprinted odors have been documented in salmon (Nevitt et al., 1994; Dittman et al., 1997). Combined, these results suggest that proliferation of olfactory receptor neurons and growth in the input layer of

the bulb may be part of the neuro-substrate for imprinting. At the level of the olfactory epithelium, the development and turnover of olfactory receptor neurons occurs by proliferation of multipotent basal stem cells (Caggiano et al., 1994; Farbman, 1994; Huard et al., 1998; Jang et al., 2003). Proliferation of these cells has been shown to be stimulated by thyroid hormones in a variety of animals, including rats (Mackay-Sim and Beard, 1987; Paternostro and Meisami, 1989, 1994) and frogs (Burd, 1990, 1992). Although it is not known whether thyroid hormones influence proliferation in the salmon olfactory periphery, the epithelium of masu salmon (*Oncorhynchus masou*) becomes enriched with thyroid hormone receptors during smolting, suggesting that olfactory tissues may be particularly sensitive to effects of thyroid hormones at this time (Kudo et al., 1994).

We have begun to test these ideas by examining whether thyroid hormones influence cell proliferation in the olfactory epithelium of juvenile coho salmon. Because T_4 is converted extrathryoidally by 5′-deiodinase to the intracellularly active form 3,5,3′-triiodothyronine (T_3) (DeGroot et al., 1978; Köhrle, 1999), we manipulated thyroid hormone levels by intraperitoneally implanting T_3 or placebo pellets for 16–20 days to mimic smolting. We then used an established 5-bromo-2′-deoxyuridine (BrdU) immunocytochemical cell birth-dating technique to compare the density and spatial distribution of mitotic cells and cell clusters within the olfactory epithelium between the two treatment groups. Finally, we explored whether natural fluctuations in plasma levels of T_4 are associated with changes in cell proliferation in the epithelium during smolting.

Materials and methods

Animal housing

Coho salmon parr (*Oncorhynchus kisutch* Walbaum; ~4 months old) were obtained from the Iron Gate Fish Hatchery located on the Klamath River, California. Upon arrival at the University of California at Davis, fish were housed at the Center for Aquaculture and Aquatic Biology Facility in four circular rearing tanks (1.2 m diameter, 380 liter capacity). Water was supplied from an on-site aquifer and delivered to tanks at a constant rate (15 l min^{-1}) throughout the experiment. Fish were maintained at 11±1°C under ambient photoperiod.

Experiment 1: thyroid hormone manipulations

Thyroid hormone levels were artificially elevated by implanting T_3 (or placebo) pellets intraperitoneally (5 mg of 3,5,3′-triiodo-L-thryonine per pellet; Innovative Research of America, Sarasota, FL, USA). Fish were sacrificed 16–20 days after pellet implantation to mimic the elevated thyroid hormones during smolting. At nine months of age, parr were lightly anesthetized (MS-222 immersion, 1:1000; Crescent Research Chemicals, Phoenix, AZ, USA) and the pellet was inserted with surgical forceps into a small (5 mm) incision in the peritoneum. The incision was sealed with all-purpose Krazy® glue (Elmer's Products Inc., Columbus, OH, USA). We measured the standard length and recorded the mass of each fish. Placebo- and T_3-implanted fish were housed separately with equal numbers of non-implanted companion fish in two identical holding tanks. Due to technical limitations, hormone levels and BrdU immunocytochemical analysis could not be performed on the same individuals. Separate fish from each treatment were thus used to measure hormone levels and to quantify cell proliferation.

Experiment 2: natural fluctuations in thyroid hormones

From 18 December 1997 to 9 April 1998, we sampled unmanipulated, juvenile salmon every two weeks to measure plasma levels of the thyroid hormones T_4 and T_3 and to quantify cell proliferation in the olfactory epithelium using the established BrdU immunocytochemical cell birth-dating technique. Separate fish from the same cohort and holding tank were used to measure hormone levels and to quantify cell proliferation for each sampling day.

Blood collection for plasma hormone analysis

Blood was collected from the caudal peduncle using a heparinized capillary tube. Blood was centrifuged, and plasma was stored at –80°C for subsequent hormone analysis. Plasma was assayed for total T_3 using a single antibody enzyme-linked immunosorbent assay (ELISA) procedure (modified from Schall et al., 1978). Binding of the free hormone to other proteins was inhibited by ANS (8-anilonaphthalene sulfonic acid). The binding of peroxide-labeled antibodies was measured by reacting the peroxide to 3,3′,5,5-tetramethylbenzidine. The reaction was terminated with HCl and read at 450 nm using a microplate reader (Bio-Tek EL311s). Each plate was prepared with five standards (0–50 ng ml^{-1}). Standard concentrations regressed against $\log_e$(OD) yielded an r^2 of >0.99.

BrdU immunocytochemistry

Fish were injected intraperitoneally with BrdU (0.05 mg g^{-1} body mass; Sigma, St Louis, MO, USA). BrdU is incorporated into replicating DNA, and the systemic application of BrdU is a well-established technique for labeling mitotically active cells in a variety of taxa including fish (e.g. Zupanc and Horschke, 1995; Ekström et al., 2001). After a survival time of 1 h, BrdU-injected fish were deeply anesthetized (MS-222 immersion, 1:1000) and perfused by intracardial injection of chilled heparinized phosphate-buffered saline (PBS; 0.1 mol l^{-1}) followed by Bouin's fixative. Olfactory rosettes and the brain were dissected and postfixed in Bouin's. At this time, the peritoneum was checked to confirm the presence of the implanted pellet in the event that it might have been exuded over the course of the experiment.

After postfixation for 12 h, tissue was dehydrated in a graded ethanol series, cleared in toluene and embedded in Paraffin. After serial sectioning (9 μm) and mounting, tissue sections were then deparaffinized and rehydrated. Chromatin was

precipitated with 2 mol l^{-1} HCl (30 min), followed by quenching endogenous peroxidase activity with 3% H_2O_2 (15 min). Tissue was rinsed between each step with PBS-D (PBS containing 1% dimethylsulfoxide; Sigma). After a 2 h blocking reaction with normal horse serum (1.125% in PBS-D), sections were incubated overnight at 4°C with primary anti-BrdU antibody solution (1:1000 PBS-D dilution; Sigma). Antibody binding was visualized by incubation with a biotinylated horse anti-mouse IgG, avidin–biotin–peroxidase complex (mouse IgG ABC Kit; Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (DAB) with nickel enhancement. Staining controls that included preincubating the primary antibody in the presence of excess BrdU prevented all immunohistochemical staining.

Quantification of BrdU-labeled cell density

The number of cells immunoreactive to BrdU was determined using computer-aided analysis (NIH Image 1.60; National Institutes of Health, Gaithersburg, MD, USA) of images captured *via* digital camera (Cohu Inc., San Diego, CA, USA) attached to an Axioskop microscope (Zeiss, Oberkochen, Germany). For each fish, we randomly selected one lamella from the rosette and counted all BrdU-immunoreactive (BrdU-ir) cells in sections every 90 μm throughout the entire lamella. The progenitor cells that differentiate into neurons in the olfactory epithelium occur in a specific cellular layer at the base of the epithelium (e.g. Caggiano et al., 1994; Huard et al., 1998). Consequently, we divided the olfactory epithelium into two regions for analysis: (1) the basal region and (2) the mid-apical region. BrdU-ir cells were classified as within the basal region when any portion of the cell was located within 10 μm of the basal edge of the epithelium; all other BrdU-labeled cells were considered within the mid-apical region. This division allowed us to distinguish between an increase in proliferation along the basement membrane of the epithelium (the basal region) and proliferation within the remainder of the epithelium (the mid-apical region).

We also quantified clusters of BrdU-ir cells. We defined a cluster as a group of at least two BrdU-ir cells that appeared to be in physical contact with each other. Results were analyzed as the number of BrdU-ir cells per length (μm) of lamella to standardize for any differences in lamellar sizes between fish.

Statistical analyses

To compare plasma T_3 levels between T_3-implant and placebo treatments, the hormone values were $\log_e$ transformed. Comparisons were then made using a two-sample *t*-test.

We analyzed the number of BrdU-labeled cells using Mann–Whitney *U*-tests to compare the number of positively immunoreactive cells between treatments (SyStat 8.0; SyStat, Inc., Point Richmond, CA, USA). Separate analyses were conducted for the clustered, single and total number of BrdU-ir cells in the basal and mid-apical regions of the olfactory epithelium. All statistical values are reported as means ± S.E.M. As we specifically predicted that elevated T_3 would induce an increase in cell proliferation (e.g. Nevitt and Dittman, 2004), all Mann–Whitney *U* statistics are one-tailed.

To determine whether cells were randomly distributed among clusters, we first tallied the occurrences of different sizes of clusters (i.e. number of BrdU-ir cells per cluster) for each individual fish from both treatment groups. We then compared the mean distribution of cluster sizes for each treatment group to one created by Poisson (random) process. Because the cluster data follow a truncated Poisson distribution (Zar, 1996), we estimated the Poisson parameter (λ) for each distribution using tables provided in Cohen (1960). We also used a χ^2 test to compare the distribution of BrdU-ir cluster sizes between treatments (Zar, 1996). Exact *P*-values were obtained using StaTable 1.0.1 (Cytel Software Corp., Cambridge, MA, USA).

We used analyses of variance (ANOVAs) to test for changes in body mass (g), standard length (mm) and condition factor {*k*; calculated as: $[(10^5)(\text{body mass})/(\text{standard length})^3]$; see LeCren, 1951} over the nine sample dates from December 1997 to April 1998. To evaluate changes in thyroid hormone levels, we first used a Bartlett's test to examine homogeneity of variances over the nine sampling days. When variances were equal among sampling dates, we used an ANOVA to test for changes in hormone levels among sampling days; when variances were unequal, a Welch test was used (Zar, 1996). All analyses were conducted using JMP 4.0.2 statistical software (SAS Institute, Inc.).

We used Kruskal–Wallis tests to examine changes in the density of BrdU-labeled cells and cell clusters over the nine sampling days. Spearman rank correlations were used to examine the relationship between mean thyroid hormone levels and mean density of BrdU-labeled cells in the epithelium among sampling dates (Zar, 1996). Because we predicted that an increase in thyroid hormone would relate to an increase in the density of BrdU-labeled cells (Nevitt and Dittman, 2004), tests for the significance of correlations were performed one-tailed.

Results

Experiment 1: thyroid hormone manipulations

Thyroid hormone levels

Fish implanted with 3,5,3′-triiodothyronine pellets showed significantly increased circulating levels of T_3 (N=15; 22.86±1.55 ng ml^{-1}) compared with fish implanted with placebo pellets (N=16; 0.79±0.19 ng ml^{-1}) (*t*-test, d.f.=29, t=6.185, P<0.0001). T_4 is the primary form of thyroid hormone secreted from the thyroid gland, and plasma levels of T_3 are low relative to T_4 (Young et al., 1989; Specker et al., 1992). Mean plasma T_4 has been recorded as high as 91 ng ml^{-1} in smolting salmon (Dickhoff et al., 1978), suggesting that the elevated hormone levels were within physiological range at the level of the target tissue. However, comparing plasma T_3 and T_4 levels is difficult, given that T_3 is the intracellularly active form of thyroid hormone and that conversion of T_4 to T_3 is

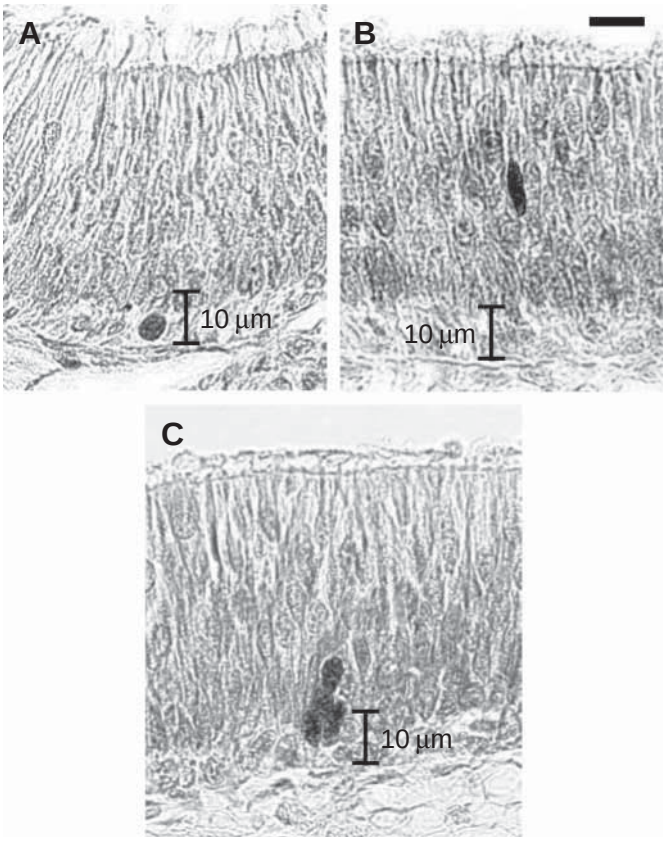

Fig. 1. Section of the olfactory epithelium showing BrdU-ir in single and clustered cells. Positive staining appears as a black precipitate. (A) Typical round morphology of a BrdU-ir cell in the basal cell layer. (B) Elongated morphology typical of a BrdU-ir cell in the mid-apical cell layer. (C) An example of a BrdU-labeled cluster of four cells situated in the basal region of the epithelium. Labeled cells are black and appear to be in close juxtaposition to each other. Length bars (10 μm) distinguish the extent of the basal region in A, B and C. Scale bar in B, 10 μm.

regulated by enzymatic deiodination at target sites (e.g. Köhrle, 1999).

Morphology and distribution of BrdU-labeled cells

BrdU-labeled cells were easily identified because they displayed a black precipitate typical of the DAB reaction product with nickel enhancement (Figs 1, 2). BrdU-ir cells were distributed throughout the basal and mid-apical regions of the epithelium. In the basal epithelium, BrdU-ir cells tended to be round (Fig. 1A). These basal cells were morphologically consistent with globose basal stem cells, which give rise to both olfactory receptor neurons and support cells (Schwob et al., 1994; Huard et al., 1998). In the mid-apical region, BrdU-ir cells tended to be more elongated (Fig. 1B). In addition to labeling single cells, BrdU labeled cell clusters. Clusters were groups of two or more cells that appeared to be in contact with each other (Fig. 1C). Although clusters were identified throughout both the basal and mid-apical regions of the epithelium, the basal region contained significantly more clusters than the mid-apical region (Wilcoxon paired-sample test, T=37, P<0.001, N=24).

T_3 increases BrdU-ir cells in the basal cell layer

Fig. 2 shows representative photomicrographs of BrdU labeling in the epithelium of both placebo (N=11; Fig. 2A–C) and T_3-implanted fish (N=13; Fig. 2D–F). Overall, the total number of BrdU-labeled cells in the olfactory epithelium was significantly greater in fish implanted with T_3 pellets (19.07±3.41 cells per 100 μm lamella length; mean ± S.E.M.) than in fish with placebos (10.48±1.65 cells per 100 μm lamella length) (Mann–Whitney U-test, U=105, P=0.026). Within the basal region, the total number of BrdU-ir cells was greater in T_3-implanted fish than in placebo fish (Fig. 3A: U=105, P=0.026). However, the number of BrdU-ir cells distributed in clusters in the basal region was not significantly different between treatments (U=74, P=0.443). Thus, the observed difference in total BrdU-labeled cells resulted from a greater number of single BrdU-ir cells in the experimental group (U=106, P=0.024). By contrast, the total number of BrdU-labeled cells in the mid-apical region did not differ between T_3 implant and control treatments (Fig. 3B; U=94, P=0.096). Both clustered (U=78.5, P=0.342) and non-clustered BrdU-ir cells (U=93, P=0.107) were just as frequent in the mid-apical layer in each treatment group. Finally, we found no evidence for a significant increase in the total number of clusters (T_3 implant, 0.111±0.024 clusters per 100 μm lamella length; placebo, 0.068±0.012; U=94, P=0.096). Likewise, the number of clusters was similar between treatments within both the basal (T_3 implant, 0.075±0.021 clusters per 100 μm lamella length; placebo, 0.046±0.008; U=83, P=0.253) and mid-apical regions (T_3 implant, 0.036±0.009 clusters per 100 μm lamella length; placebo, 0.022±0.006; U=86.5, P=0.192). Even though we found significant differences in the density of BrdU-labeled cells, this change in the rate of cell proliferation did not translate into a difference between treatments in length of the olfactory lamella (t-test, d.f.=22, t=–0.151, P=0.8815).

Evidence for targeted proliferation of clusters

Distributions of cluster sizes differed from a Poisson (random) process for both T_3 and placebo treatments (Fig. 4; T_3 implant, χ^2=12.6697, P=0.0004; placebo, χ^2=7.0978, P=0.008), suggesting that, in each case, certain clusters were targeted to proliferate. We next compared distribution patterns between treatment groups to examine potential influences of T_3 on this process. We found that, while large clusters seemed to occur more frequently in the epithelium of T_3-implanted fish (Table 1), the overall distribution of cluster sizes did not differ

Table 1. *Numbers of fish from the T_3-implant and placebo treatments with clusters containing* ‘x’ *or more cells*

		Cluster size ($\geqslant x$)				
	N	$\geqslant$3	$\geqslant$4	$\geqslant$5	$\geqslant$6	$\geqslant$7
T_3 implant	13	13	8	4	2	1
Placebo	11	10	6	1	0	0

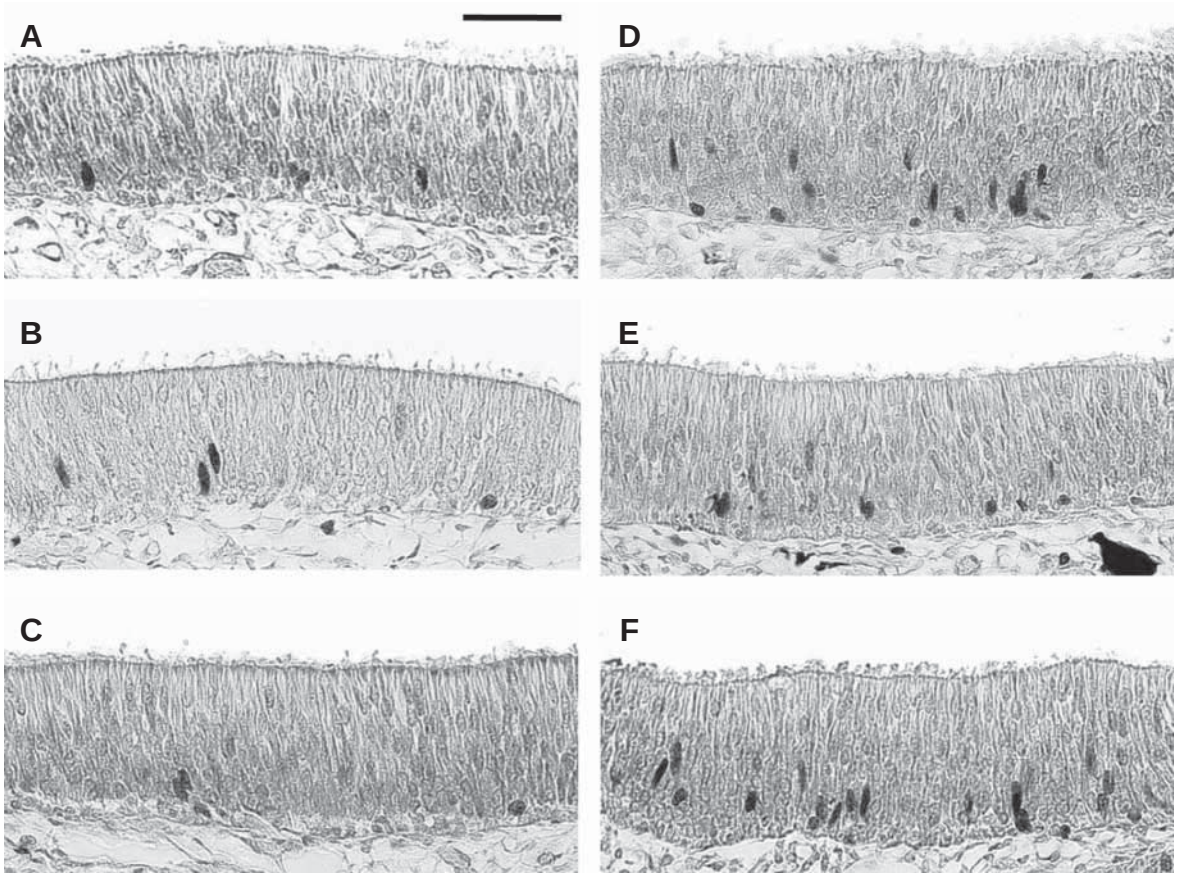

Fig. 2. Representative BrdU labeling in the olfactory epithelium of placebo fish (A–C) and T_3-implanted fish (D–F). Photomicrographs are shown from six different individual fish. Scale bar, 40 μm.

significantly between T_3 and placebo treatments (Fig. 5; χ^2=0.8289, d.f.=4, P=0.9345), suggesting that targeted proliferation was not influenced by T_3.

Experiment 2: natural fluctuations in thyroid hormones

Morphological changes during smolting

Over the course of this study, fish lost their parr marks and turned a silvery color characteristic of smolting. In addition, fish nearly doubled their body mass (Fig. 6A; ANOVA, $F_{8,91}$=8.1153, P<0.0001) and increased their body length by approximately 20% ($F_{8,91}$=10.2666, P<0.0001). Condition factor (k), an indication of fish body status or 'fatness' (LeCren, 1951), also showed significant changes from December 1997 to April 1998 (Fig. 6B; $F_{8,91}$=3.0190, P=0.0047). Pairwise comparisons (Tukey HSD test, overall α=0.05) indicated a significant difference between condition factors calculated for 3 January 1998 and 7 April 1998, suggesting a decreasing trend over the four-month sampling

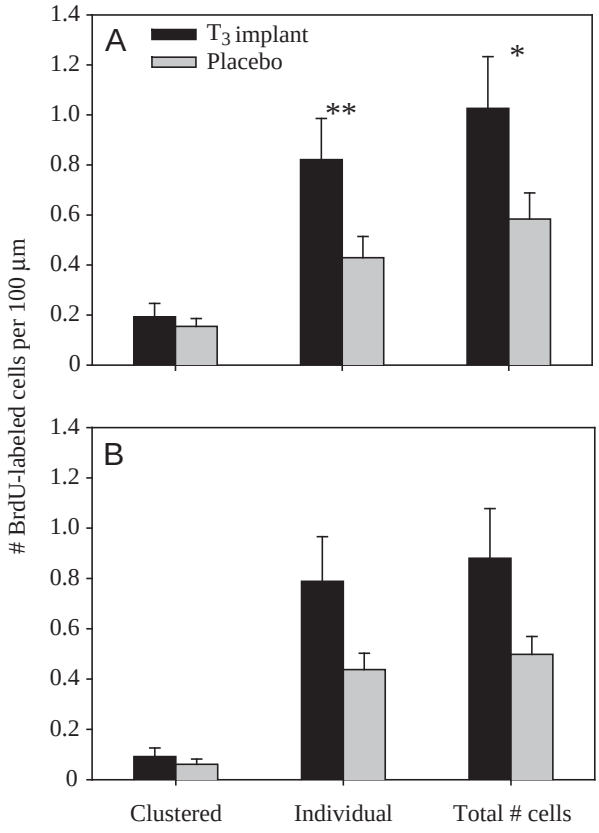

Fig. 3. Counts of BrdU-ir cells in the basal (A) and mid-apical (B) cell layers of the olfactory epithelium of T_3-implanted (black bars) and placebo (gray bars) fish. Clustered and individual cells are considered separately in the first two columns, and together in the third column ('total cells'). Values are expressed as means ± S.E.M. Mann–Whitney U-tests: ∗P<0.05; ∗∗P<0.025.

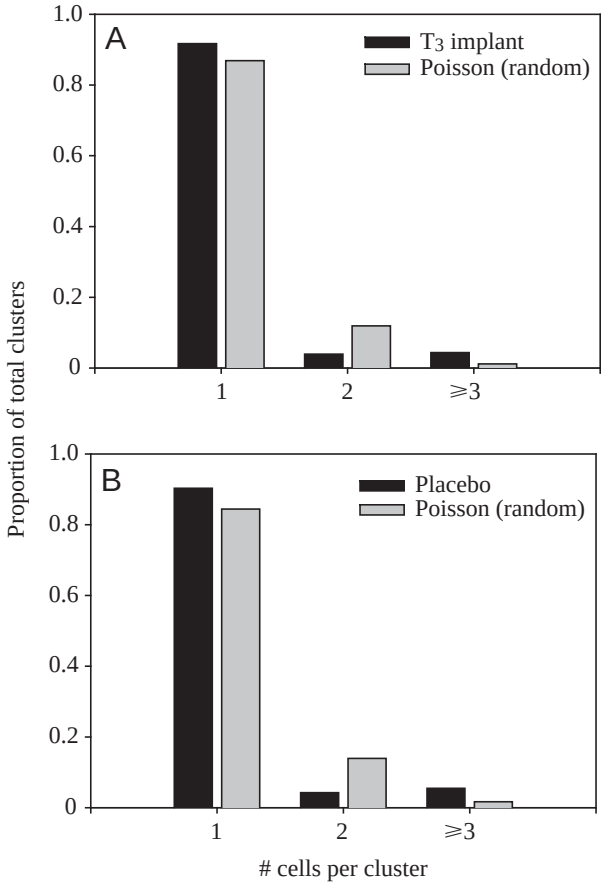

Fig. 4. The distribution of cluster sizes for each treatment group compared with a Poisson (random) distribution. The frequency of cluster sizes for the (A) T_3-implanted group (black bars) and the (B) placebo group (black bars) compared with a frequency distribution generated by a truncated Poisson process (gray bars in A and B). Both distributions are significantly different from random (T_3 implant, χ^2=12.6697, P=0.0004; placebo, χ^2=7.0978, P=0.008).

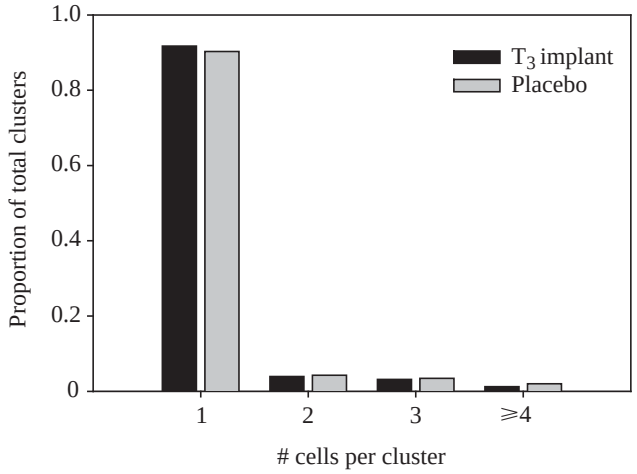

Fig. 5. The distribution of cluster sizes from T_3-implanted (black bars) and placebo (gray bars) groups. Distributions are not significantly different from each other (χ^2=0.8289, P=0.9345).

period. Taken together, the change in body coloration, increases in body mass and length, and the decrease in condition factor suggest that fish transitioned from the parr to smolt life stages over the course of the experiment (e.g. Young et al., 1989).

Plasma levels of thyroid hormones

Fig. 7 shows profiles of the thyroid hormones T_4 and T_3 for the nine sample dates spanning the four months of this study. During this time, T_4 levels fluctuated between approximately 10 and 20 ng ml^{-1} without an obvious surge (ANOVA, $F_{8,34}$=0.9853, P=0.4643). Plasma levels of T_3 were consistently low (range, ~0.45–0.90 ng ml^{-1}) relative to T_4 concentrations throughout the duration of sampling and similarly showed no statistically significant changes over the sampling period (Welch test, F=0.8308, P=0.5898).

Relationship between T_4 and cell proliferation

Fig. 8 shows profiles of mean plasma T_4 levels relative to the number of BrdU-labeled cells per micron in the basal and mid-apical regions of the olfactory epithelium. While the mean number of labeled cells fluctuated, there were no significant differences in BrdU labeling among sampling dates in either the basal (Kruskal–Wallis test, d.f.=8, H=8.428, P=0.393) or the mid-apical (H=9.327, P=0.315) regions of the olfactory epithelium. Linear regression of mean number of BrdU-labeled cells against body mass showed no significant relationship in either the basal region (r^2=0.048, P=0.572) or the mid-apical region (r^2=0.123, P=0.355) of the epithelium. Given that fish doubled in body size over this sampling period, cell proliferation in the epithelium appeared to be unaffected by the rapid growth of the fish.

BrdU-ir cell counts were significantly correlated to plasma T_4 levels. This positive relationship was restricted to the basal epithelium (Fig. 9A; clustered and single cells considered together; Spearman rank correlation, N=9, r_s=0.600, P=0.05) and was not expressed in the mid-apical region (Fig. 9B; r_s=0.233, P>0.25). The positive correlation between plasma T_4 and overall cell proliferation appears to be due to a strong relationship between plasma T_4 and the density of single BrdU-labeled cells in the basal epithelium (Fig. 9C; r_s=0.683, P<0.05). No significant relationship was found between T_4 and single BrdU-labeled cells in the mid-apical region (Fig. 9D; r_s=0.433, P>0.05). Likewise, there was no significant relationship between T_4 and the number of BrdU-labeled clusters in either the basal (r_s=0.433, P>0.05) or mid-apical (r_s=0.333, P>0.10) regions of the olfactory epithelium.

Discussion

Thyroid hormones induce basal cell proliferation in the epithelium

We observed significant increases in both the number of single BrdU-labeled cells and the total number of BrdU-ir cells within the basal region in response to artificially elevated plasma T_3 levels. These changes occurred only in the basal

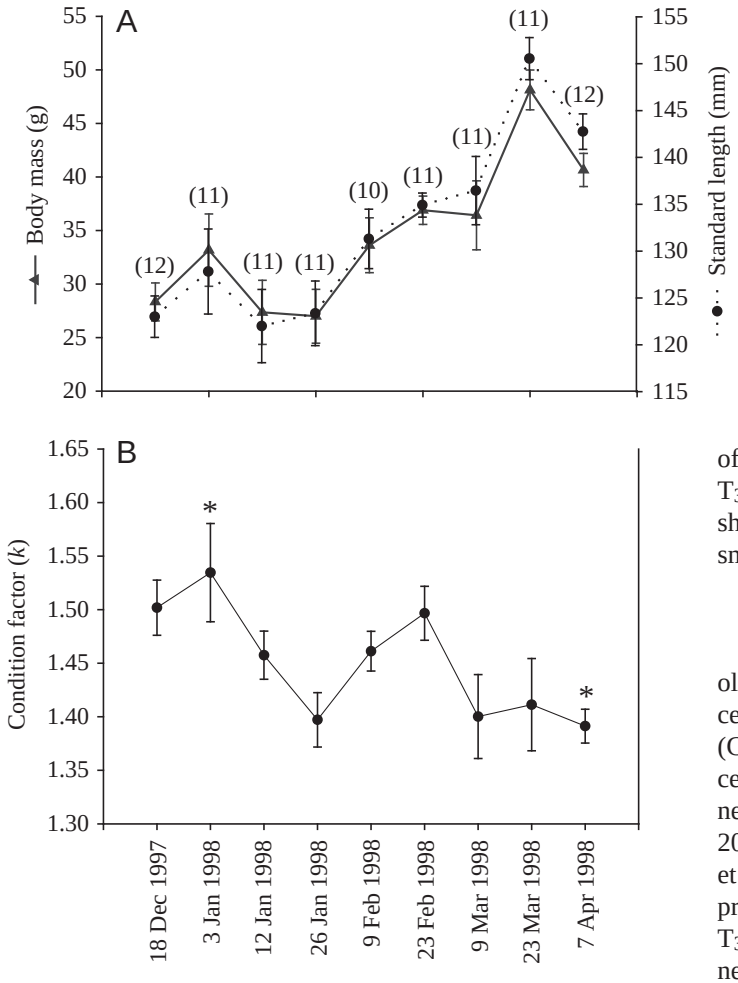

Fig. 6. (A) Profiles of body mass (triangles) and standard length (circles) plotted against sampling date. Data are given as means ± S.E.M. Numbers of fish sampled are shown in parentheses. (B) Condition factor (see Materials and methods) plotted against sampling date. Data are plotted as means ± S.E.M. Sample sizes are identical to those shown in A. Condition factor significantly decreased during the sampling period, which is a trend associated with smolting (asterisks indicate significant difference at overall $P<0.05$ between dates, Tukey HSD test).

region of the olfactory epithelium; we found no significant increases in the density of BrdU-labeled cells in the mid-apical region. We also report a significant positive relationship between plasma T_4 and the density of BrdU-labeled cells in the basal region but not in the mid-apical region of the olfactory epithelium. These results suggest that even small fluctuations in plasma thyroid hormone are reflected in changes in cell proliferation in the olfactory epithelium of salmon. Changes in proliferation are thus consistent between fish with experimentally manipulated levels of thyroid hormones and fish that are naturally smolting.

In addition to labeling single cells, we observed labeling in cell clusters (see also Moulton et al., 1970; Graziadei and Monti Graziadei, 1979; Suzuki and Takeda, 1993; Huard and Schwob, 1995; Weiler and Farbman, 1997). Weiler and Farbman (1997) have hypothesized that this clustering results from a local stimulus activating the cell cycle in several neighboring progenitor cells. If this is the case, then some clusters exposed to the local stimulus should be expanding at a faster rate than others that are either not exposed or are unresponsive to the stimulus. Our sample size (overall N=24 fish) was large enough to test this idea statistically, and our results support this idea. We found that the distributions of cluster sizes differed significantly from random regardless of treatment, indicating that there were more large clusters than expected by chance. However, although we noted large (>5 cells) clusters more frequently in T_3-implanted than in placebo fish, we found no difference in the distribution of cluster sizes among treatments. This analysis suggests that T_3 promotes a uniform increase in proliferation rather than a shift in the distribution of cluster sizes toward either larger or smaller clusters.

Identity of the proliferating cells

Our current understanding of neuronal proliferation in the olfactory system is that the basal region contains globose basal cells that act as multipotent progenitors within the epithelium (Caggiano et al., 1994; Huard et al., 1998). These globose basal cells become mitotic and give rise to both olfactory receptor neurons (Caggiano et al., 1994; Schwob et al., 1994; Jang et al., 2003) and non-neuronal cells such as sustentacular cells (Huard et al., 1998). It is thus likely that part of the increased proliferation of basal cells that we see in response to elevated T_3 represents the enhanced production of olfactory receptor neurons. This interpretation is consistent with other studies in salmon showing targeted growth in the olfactory nerve and glomerular layer coincident with the developmentally regulated surges in thyroid hormone during the parr–smolt transformation. For example, studies in Atlantic salmon suggest a quadrupling of olfactory receptor cell number, as well as specific changes in the relative composition of the olfactory bulb neuropil during this transition (Bowers, 1988). More extensive investigation of Chinook salmon (*O. tshawytscha*) confirmed these findings suggesting growth in the input layer of the olfactory bulb coincident with smolting (Jarrard, 1997).

As a cautionary note, however, it has also recently been argued that BrdU labeling may not be specific to cells in the process of mitosis (e.g. Gould and Gross, 2002; Rakic, 2002); BrdU may in fact label any cell that is undergoing DNA synthesis. It is thus possible that some of these BrdU-ir cells are not mitotic but are in the process of DNA repair. This intriguing explanation may account for the BrdU labeling we and others (e.g. Weiler and Farbman, 1997) have observed in the mid-apical layer of the epithelium where mature olfactory receptor cells are much more numerous than stem cells. Since the survival time following BrdU injection was only 1 h, it is doubtful that these elongated cells represent developing olfactory receptor neurons labeled in the basal region during S phase. A more likely possibility is that these mid-apical cells are non-neuronal sustentacular cells. While beyond the scope

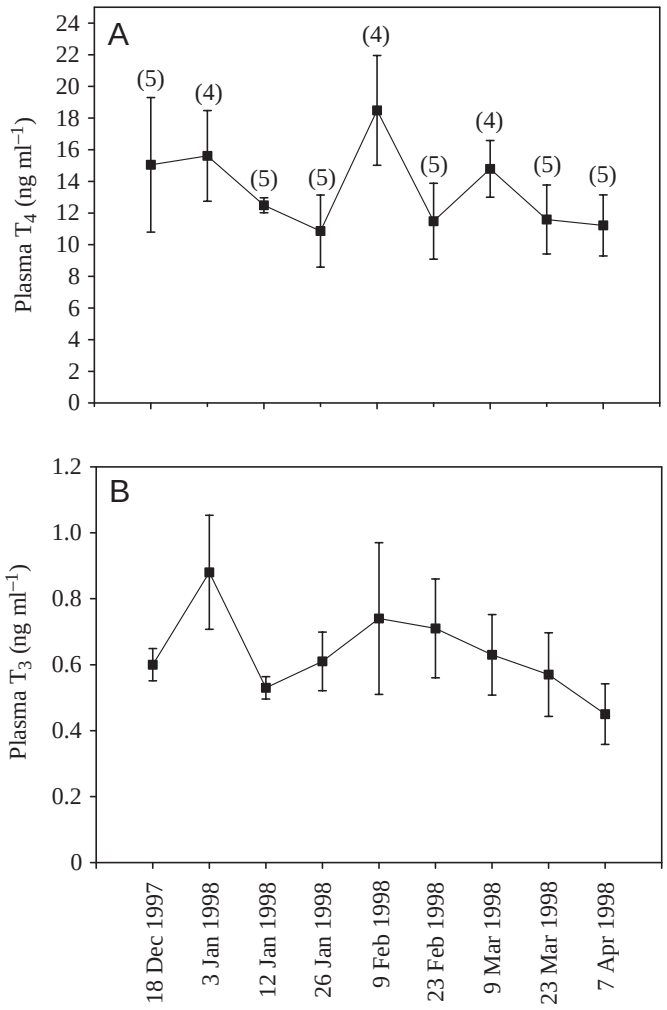

Fig. 7. Profiles of plasma T_4 (A) and T_3 (B) across the parr–smolt transformation. Data are plotted as means ± S.E.M. Sample sizes for each date are shown in parentheses in A.

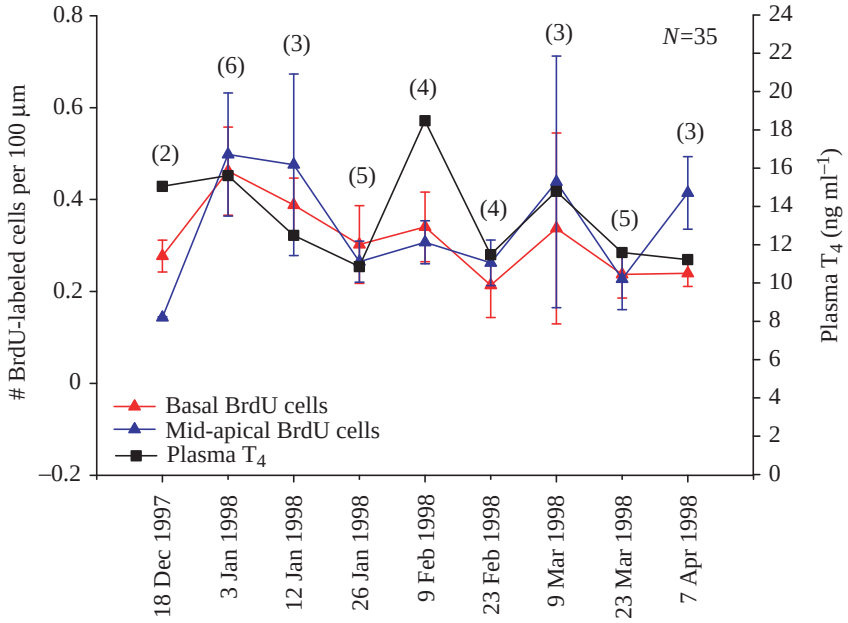

Fig. 8. Mean number of BrdU-ir cells (per 100 µm lamella length) plotted against sampling date. Data are given as means ± S.E.M. Basal cell counts are indicated by red triangles; mid-apical cell counts are indicated by blue triangles. Data are superimposed on a plot of mean plasma T_4 levels for each sampling date (black squares). Sample sizes for BrdU-ir cell data for each date are shown in parentheses.

of the current study, double-labeling with a neuron-specific marker may help to determine their identity in the future (but see Rakic, 2002).

Timing of olfactory imprinting

Our results examining naturally smolting fish suggest that even small fluctuations in plasma T_4 are associated with increased rates of proliferation of the olfactory epithelium. This result suggests that even subtle changes in the thyroid axis may influence growth in the peripheral olfactory system. This relationship may in part explain why hatchery-reared and wild salmon appear to imprint at different life stages (reviewed by Dittman and Quinn, 1996). In the wild, juvenile salmon leave the natal stream soon after emergence and often smolt a considerable distance from where they hatched. Yet these fish imprint on the natal stream and home to it as adults – not to the location where they smolted. For example, wild Kokanee salmon (the non-anadromous form of sockeye salmon, *Oncorhynchus nerka*) have been shown to imprint to artificial odorants during the alevin and fry life stages (Tilson et al., 1994, 1995). Thus, the sensitive period for imprinting appears to be more variable than suggested by the classic studies of Hasler and Scholz (1983).

A partial resolution to this paradox can be found in evidence that the thyroid axis of fish is functional early in life (for review, see Power et al., 2001). T_3 concentrations in fertilized eggs have been reported as high as 52 ng g^{-1} body mass for salmonids (Mylonas et al., 1994), and maternal thyroid receptor transcripts have recently been identified in developing eggs and alevins (Jones et al., 2002). In addition, fluctuations in thyroid hormone have been linked to both hatching and emergence of fry from the gravel streambed (Sullivan et al., 1987; Dickhoff and Sullivan, 1987; Leatherland et al., 1989; Tagawa and Hirano, 1987, 1989). There is also growing evidence that novel stimuli encountered by juvenile salmon as they emerge from the gravel, establish territories, forage and migrate downstream can stimulate increases in circulating levels of thyroid hormones. For example, the thyroid axis is sensitive to a variety of environmental cues including changes in lunar phase (Grau et al., 1981), photoperiod (Hoar, 1976; Iwamoto, 1982), water temperature (Iwamoto, 1982; Lin et al., 1985) and changes in water flow rates (Youngson and Simpson, 1984; Lin et al., 1985). And exposing salmon to novel water

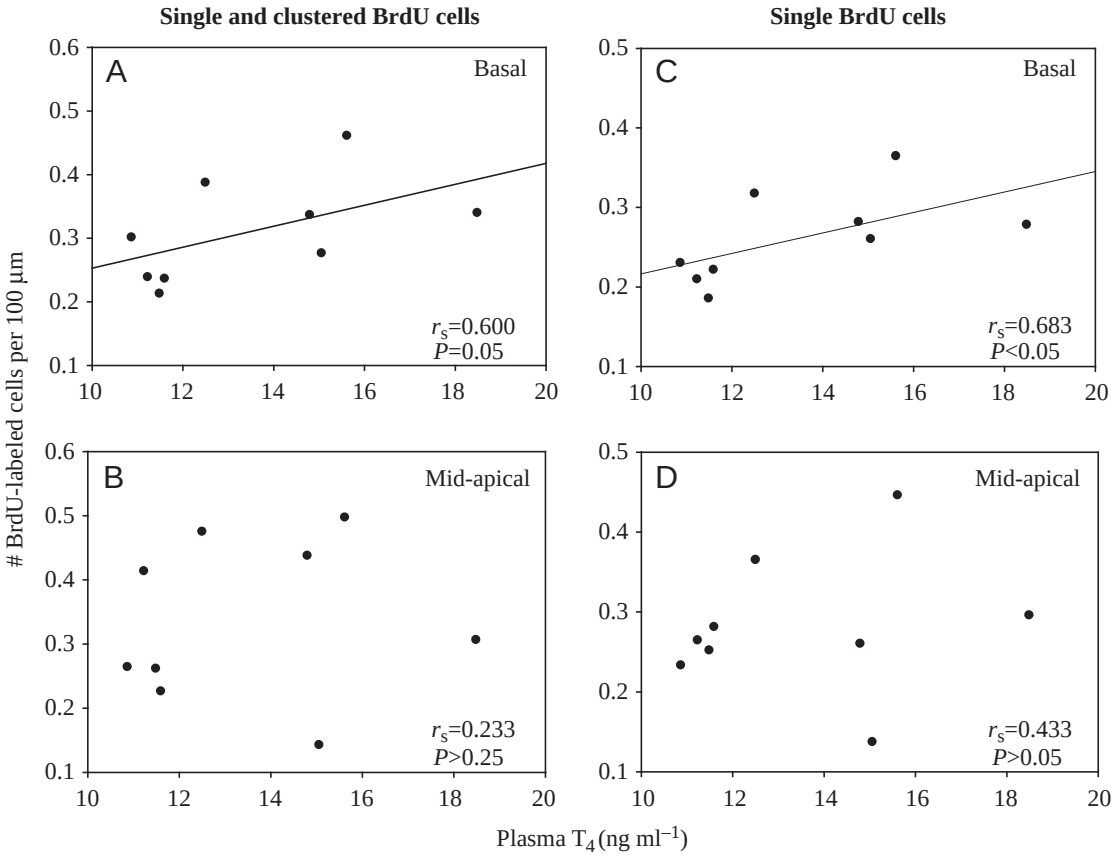

Fig. 9. Mean number of BrdU-ir cells (per 100 μm lamella length) plotted against mean plasma T_4 levels for each sampling date. (A,B) Single and clustered BrdU-ir cells considered together. The data indicate a significant positive correlation in the basal cell layer (A) but not in the mid-apical cell layer (B), suggesting that T_4-induced proliferation is restricted to the basal cell layer. (C,D) Single BrdU-ir cells only. The data show a significant positive correlation in the basal cell layer (C) but not in the mid-apical cell layer (D).

sources can alter thyroid activity (Dickhoff et al., 1982; Nishioka et al., 1985; Hoffnagle and Fivizzani, 1990).

If the peripheral olfactory tissue is competent to respond to these hormonal fluctuations, then variation in environmental conditions should lead to subtle changes in cell proliferation in the olfactory epithelium. In the case of hatchery-reared fish, this process is co-opted by smolting, simply because the over-riding consistency of the hatchery environment fails to stimulate the thyroid axis at other times of development (Dittman and Quinn, 1996). Thus, the parr–smolt transformation becomes the sensitive period for imprinting, not because fish are specifically adapted to learn odor cues during this stage of life but because the hatchery environment dampens olfactory learning during earlier life stages.

Local influences of T_4 are also regulated in part by enzymatic deiodination of thyroid hormone at the target tissue (e.g. Eales and Brown, 1993; Köhrle, 1999). While we did not address deiodinase activity here, recent work suggests that the olfactory epithelium of salmonids is less tightly regulated to respond to circulating thyroid hormones than other sensory targets (e.g. the retina; see Plate et al., 2002). The olfactory epithelium may thus be better able to mirror subtle changes in plasma T_4 triggered by changes in the fish's immediate environment. Whether this process extends to other parts of the brain potentially involved in imprinting (e.g. olfactory bulb and telencephalon; Kihslinger et al., 2003) will be an interesting area for further research.

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