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**Behavioral and biochemical mechanisms of olfactory imprinting
and homing by coho salmon**

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University of Washington, 1994

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Behavioral and Biochemical Mechanisms of Olfactory
Imprinting and Homing by Coho Salmon

by

Andrew Harris Dittman

A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

University of Washington

1994

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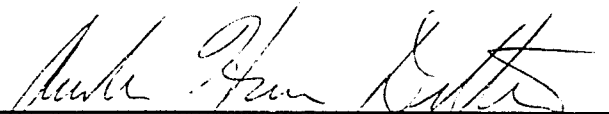
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Abstract

Behavioral and Biochemical Mechanisms of Olfactory
Imprinting and Homing by Coho Salmon

by Andrew Harris Dittman

Chairperson of the Supervisory Committee: Professor Thomas P. Quinn
School of Fisheries

The final freshwater phase of the Pacific salmon's homing migration is governed primarily by the olfactory discrimination of homestream water. Prior to their seaward migration, juvenile salmon learn site-specific odors associated with their home stream and later use these odor memories for homing. Experimental evidence suggests that olfactory imprinting by salmon occurs during a sensitive period associated with surges in plasma thyroxine levels during smolting. Life-history studies, however, suggest that imprinting may occur prior to smolting. To examine the timing of olfactory imprinting, hatchery-reared coho salmon were exposed to site-specific or artificial odorants at specific developmental stages and their subsequent abilities to respond to these odors as adults were tested behaviorally. These studies indicated that smolting may be a particularly important period for olfactory imprinting but that salmon reared exclusively in a hatchery may have impaired imprinting ability. Further studies indicated that rapidly rising plasma thyroxine is not required for imprinting but that migration may play an important role. The behavioral responses of precociously mature salmon to a putative pheromone was also examined.

The biochemical mechanisms underlying olfactory recognition of amino acids and imprinted odorants was also examined. Adenylyl and guanylate cyclase in imprinted coho salmon olfactory cilia were characterized and their roles in olfactory signalling were examined. The properties of adenylyl and guanylate cyclase were consistent with a role in olfactory transduction. Adenylyl but not guanylate cyclase was stimulated by high concentrations of amino acid odorants. Both enzymes were slightly stimulated by the imprinted odorant phenylethyl alcohol but a difference in enzyme sensitivity between imprinted and unimprinted fish was only observed with guanylate cyclase activity in

maturing fish. In higher vertebrates, olfactory transduction is mediated by the type III adenylyl cyclase enriched in olfactory cilia. The regulation of this enzyme and other adenylyl cyclase isoforms by the m4 muscarinic receptor was also examined. Activation of m4 muscarinic receptor is thought to inhibit adenylyl cyclase activity but co-transfection studies indicated that crossover from inhibitory to stimulatory G protein coupling can occur.

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LIST OF ABBREVIATIONS

BAPTA/AM	1,2-bis(2-amino-5,5'-difluorophenoxy)-ethane N,N,N',N'-tetrakis (acetoxymethyl) ester
BBC	Big Beef Creek
Ca ²⁺	calcium
CaM	calmodulin
cAMP	adenosine 3', 5'-cyclic monophosphate
cGMP	guanosine 3', 5'-cyclic monophosphate
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediamine-N,N,N',N'-tetraacetic acid
EGTA	[ethylene bis (oxyethylenenitrilo)tetraacetic acid
FW	fresh water
G protein	guanine nucleotide binding protein
GDP-β-S	guanosine-5'-O-(2-thiodiphosphate)
GtH	gonadotropin
GTP	guanosine 5'-triphosphate
GppNHp	5'-guanylyl-imidodiphosphate
HEK 293	human embryonic kidney 293 cells

IBMX	3-isobutyl-1-methylxanthine
IP3	D-myo-Inositol 1,4,5-triphosphate
Lubrol PX	polyoxyethylene (9) lauryl ether
PEA	phenylethyl alcohol
PI	phosphoinositide
PMSF	phenylmethanesulfonyl fluoride
PTX	pertussis toxin
[3H] QNB	1-Quinuclidinyl[phenyl-4-3H]benzilate.
SC	Seattle city
SP	Seward Park
SW	sea water
T3	triiodothyronine
T4	thyroxine
TCA	trichloroacetic acid
Tris-HCL	tris (hydroxymethyl) amino methane hydrochloride
UW	University of Washington
17 α , 20 β -P	17 α , 20 β -dihydroxy-4-pregnene-3-one

ACKNOWLEDGEMENTS

My thesis work has involved many areas of research and would not have been possible without the help and support of many students, faculty and staff in both the Pharmacology Department and the School of Fisheries. Specifically, I would like to thank my thesis advisors Tom Quinn and Dan Storm. Tom has been both mentor and friend to me and I will always cherish the opportunity I have had to work with him. Similarly, I especially want to thank Dan Storm for giving me the opportunity to do all of the biochemical work described here and for taking me in when I was wandering the halls of the Health Sciences building looking for someone interested and willing to give a fish guy a chance. I would also like to thank all the members of my committee, especially Walt Dickhoff, Neil Nathanson, and Bill Moody for their helpful discussions. I am forever indebted to Blake and Joyce Smith and Jeff and Ahn Silverstein for helping keep my fish alive but mostly for being good people and good friends. I would also like to thank the mother of my fish and soulmate in the salmon nose crusade, Gabby Nevitt, for all her help and encouragement. I am grateful to the many other people who helped me in the field and with rearing fish, especially Glenn Yokoyama, Tru the Vu, Bill Hershberger, Tim Linley, Ernie Brannon, Jim Meyers, John Konecki, Alan Olsen and Greg Blair. I would like to thank all the members of the Storm lab, past and present for their help, ideas and criticisms but especially for making the lab a fun place to work. Finally I would like to thank my wife Stephanie for her never ending support, love, patience and encouragement. During the course of my dissertation I was supported by a NIH training grant NIEHS ES07032 and the research was supported by NIH grants NS20498 and HL44948 to Dan Storm and NSF grants BNS 8908697 and 8732224 to Tom Quinn.

DEDICATION

Dedicated to my wife, Stephanie; my parents, William and Catherine Dittman; and my Grandmother Katie Harris.

Chapter I

Timing of Imprinting and Homing to Site-specific and Artificial Odors by Coho Salmon

Introduction

Salmonids display many different life history patterns but homing to the natal site prevails throughout the family. They may be iteroparous or semelparous, rear exclusively in freshwater or be anadromous, and migrate to their natal river months before maturation or just prior to spawning (Groot and Margolis 1991). There is extraordinary diversity of freshwater habitats utilized and migratory patterns within and among salmonid species. For example, upon emergence from their natal gravel, juvenile salmon may migrate immediately to sea (e.g. chum, *Oncorhynchus keta*; pink, *O. gorbuscha*), remain in the stream (e.g. coho, *O. kisutch*; chinook, *O. tshawytscha*; steelhead, *O. mykiss*; cutthroat trout, *O. clarki*; Atlantic salmon, *Salmo salar*) or migrate and rear in lakes (sockeye, *O. nerka*). Juveniles from different populations of chum salmon may emerge directly into estuarine water or may migrate 1000's of km to sea. The generality of homing in salmonids in light of such variation in life histories and habitats suggests that the mechanisms involved in acquiring site-specific cues and subsequent use of these cues to guide the homing migration must be both spatially and developmentally flexible.

The freshwater phase of the salmon's homing migration is governed primarily by the olfactory discrimination of home stream water. Prior to or during their seaward migration, juvenile salmon learn odors associated with their natal stream and later use these odor memories to guide their migration back to their river of origin for spawning (reviewed in Hasler and Scholz 1983). Based on a series of classic experiments, Hasler and Scholz (1983) hypothesized that juvenile salmon imprint on their homestream odors during a single sensitive period associated with smolting (the physiological, morphological and behavioral transformation that readies salmon for life at sea). Many of these adaptations are associated with surges in the hormone thyroxine (T4) that occur during smolting (Dickhoff et al. 1978; Dickhoff and Sullivan 1987) and these high T4 levels are thought to be involved in olfactory learning. Coho salmon smolts exposed to synthetic chemicals such as morpholine or phenylethyl alcohol (PEA) were attracted to

unfamiliar streams scented with these chemicals during their spawning migration 1.5 years later (Cooper et al. 1976; Scholz et al. 1976). Subsequent laboratory experiments indicated that pre-smolt salmon were unable to learn these odorants unless they were injected with thyroid stimulating hormone to artificially raise T4 levels and induce premature smolting (Scholz 1980; Hasler and Scholz 1983). In these experiments, odorants were only recognized and attractive to fish subsequently injected with gonadotropin to induce maturation (Hasler and Scholz 1983). A link between smolting, thyroid activity and olfactory imprinting has also been observed for Atlantic salmon (Morin et al. 1989a,b) but coho salmon can imprint without experiencing high or rapidly increasing levels of plasma T4 (Chapter III).

Experimental evidence with hatchery or laboratory-reared fish indicates that the smolting period is important for imprinting but the complex freshwater rearing patterns of wild salmon suggest that olfactory imprinting must also occur prior to smolting. For example sockeye salmon fry emerge from their natal stream gravel (often lake tributaries) and immediately migrate into rearing lakes where they live for up to two years before smolting and migrating to sea. During their homing migration, adults migrate past the outlet streams and lakes experienced as smolts and return to their natal stream to spawn. Similarly, coho and chinook salmon may make extensive movements in freshwater prior to smolting (e.g., Peterson 1982; Murray and Rosenau 1989) yet home to their natal site.

The natural migratory patterns of juvenile salmon in freshwater and subsequent homing to the natal stream led Harden Jones (1968) and Brannon (1982) to propose that juvenile salmon learn a series of olfactory waypoints as they migrate through freshwater and later retrace this odor sequence as adults. Such a scenario might explain the mixed effects on homing that are observed when salmon are transported from their rearing site and released at a second site. Juvenile salmon transported to a different river system or long distances downstream within the same river system tend to return as adults to their site of release (e.g., Donaldson and Allen 1957; Jensen and Duncan 1971, Solazzi et al. 1991; reviewed in Lister, 1981). On the other hand, the closer the release site is to the rearing site, the higher the percentage of salmon returning to their rearing site as adults (Lister et al. 1981; Johnson et al. 1990) suggesting that salmon may initially return to their site of release but if they can detect the odors of their rearing site they will continue on to this site. This hypothesis was supported by our earlier finding that coho salmon smolts displaced downstream prior to release were able to learn the odor characteristics of both their rearing site and their downriver release site (Quinn et al. 1989).

Most studies of the timing of imprinting for homing have focused on the ability of juveniles to learn site-specific odors just prior to or during smolting (Hasler and Scholz 1983). However, soon after emergence salmon learn odors associated with specific habitats (e.g., lake vs. river water) (Brannon 1972; Bodznick 1978) and odors from other fishes (e.g., Quinn and Hara 1986; Courtenay 1989). Morphological evidence suggests that the salmonid olfactory system is functional as early as hatching (Brannon 1972; Hara and Zielinski 1989). Courtenay (1989) demonstrated that juvenile coho salmon exposed to morpholine shortly after or even before hatching, retained a memory of this odorant over a year later. It is not known whether odor memories established in early development are maintained until maturity and used for homing. In the present study, we tested whether salmon exposed to odors at early developmental stages would subsequently use these odor cues as mature adults during homing. Specifically, we examined the timing of olfactory imprinting by exposing juvenile salmon to odors as alevins, parr and smolts or continuously throughout their life and subsequently testing the responses of mature and immature fish to these odors 1.5 years later.

Methods

All experiments were conducted using fish from the University of Washington's (UW) population of coho salmon. They are reared under an accelerated feeding and temperature regime which allows them to undergo smolting in the first spring after hatching (Brannon et al. 1982). Three experimental groups of salmon were established to test the ability of juvenile salmon to learn chemical cues during distinct developmental stages. The first "migratory" group was reared and released at the Seward Park (SP) hatchery upstream from UW (Fig. 1) except for brief exposure to site-specific odors at the UW hatchery during specified developmental stages. Upon release as smolts these fish migrated to sea and their return patterns were examined in 1989 and 1990. Adult salmon returning to the Lake Washington watershed must pass within 100 m of the UW hatchery to reach Seward Park hatchery, hence we hypothesized that salmon which had learned the odors of UW during brief exposures might return there. The second "PEA" group was exposed to the artificial odorant PEA at the alevin, parr and smolt stages and was retained at the SP hatchery. The responses of mature and immature fish to PEA were examined in two-choice channel experiments conducted in 1989. A final group of salmon

(continuous-exposure group) was exposed to SP water throughout their life and their responses to SP water upon maturation were determined.

Migratory group

On November 30, 1987 we collected, pooled and fertilized 80,000 eggs from adult coho salmon that had returned to spawn at the UW hatchery. These eggs were initially reared communally at UW in dechlorinated Seattle city water. City water is drawn from the Tolt River reservoir located outside the Lake Washington watershed and was not discharged during the period when adults were returning in 1989 and 1990. Previous experiments have demonstrated that adult coho salmon can distinguish between city water and normal hatchery water (Quinn et al. 1983) and we assumed no olfactory learning would occur prior to the eyed stage. On January 7, 1988, 60,000 eyed eggs were transferred to the SP hatchery for further incubation. The remaining 20,000 eggs at UW were switched from city water to normal UW water, supplemented with water pumped from the hatchery's adult return pond and designated the egg/alevin exposure group. At both hatcheries, all eggs hatched by late January and yolk absorption was complete by late February. On March 13, egg/alevin exposure fish were transferred to SP and reared without further exposure to UW water. On May 9, 1988 10,000 fish from SP were transferred and exposed to UW hatchery/homing pond water until May 20. These fish, designated the parr exposure group, were transferred back to SP and reared until release without further exposure to UW water. The final UW group was exposed to UW hatchery/homing pond water as smolts from June 15-25 and returned to SP for final rearing. Fish which only experienced city water and SP were maintained as a non-UW exposure control. Smolting was determined by morphological changes and osmoregulatory capabilities as described below. Prior to release fish which had not smolted as determined morphologically were culled and the remaining fish in each treatment group were given a distinct coded wire tag and released into Lake Washington at the SP facility on July 3, 1988. Approximately 7000 fish per group were released at an average weight of 17.53 g.

Although adult UW coho salmon return primarily in the second year after their release to the Lake Washington watershed (1989 for this study), we monitored both the UW and Seward Park hatchery for returning coded-wire tagged fish from 1988 through 1990. The number and location of experimental fish captured in commercial and sport fisheries was also examined.

PEA-exposure group

On December 1, 1987, 20,000 coho salmon eggs were pooled, fertilized, and divided into four lots at the UW hatchery. Each egg lot was reared separately in UW water and hatching was completed by late January. The alevin, parr and smolt exposure groups were continuously exposed to 10-7 M PEA from Jan. 29-Feb. 20, May 9-20, and June 15-24, respectively. A fourth group was maintained as a PEA-naive control. Smolting status was determined by morphological changes and the ability of fish to regulate their plasma sodium levels when challenged with sea water (see below). After smolting, each group was given a distinct fin clip, transferred to the SP hatchery and reared communally to maturity (fall 1989).

I. Big Beef Creek Experiment

Behavioral experiments were conducted from Nov. 11 to Dec. 20, 1989 in a two-choice arena constructed in a controlled flow spawning channel located at the UW Big Beef Creek (BBC) Research Station, Kitsap Peninsula, Washington (Fig. 2). BBC is outside the Lake Washington watershed and its water was unfamiliar to all experimental fish. The experimental arena consisted of two contiguous gravel-lined sections: a large upstream section (23.7 x 6.2 m, 0.35 m water depth) separated by a low (0.2 m) waterfall from a smaller downstream section (15.3 x 1.4 m, 0.75 m water depth). The upstream section was divided into two arms by a 10.0 m concrete barrier extending downstream from the channel inlet. Funnel traps constructed in each arm allowed fish to move upstream into either arm but did not allow them to leave. Water for the arena was taken from BBC at a point 0.4 km above the channel inlet. During the course of the study, water flow in each arm varied between 50 and 70 l/sec but did not differ between arms.

On Nov. 11, 1989, 25 mature tagged salmon from each group were transported from Seward Park hatchery and released into the downstream section of the experimental arena. Fish were allowed to move freely within the arena until they entered a trap. Each day trapped individuals were removed, identified and their arm choice was recorded. Twice a week, new fish from each experimental group were released into the arena to maintain the original density. We continued this procedure until 110 fish from each experimental group had been tested. From Nov. 11 to Nov. 14, 1989, no PEA was metered into the arena and fish making choices during this period were designated as "no

PEA present" controls. Between Nov. 15 and Dec. 20, PEA was continuously metered into the inlet of arm B of the arena and adjusted daily to give a concentration of 10^{-7} M PEA. The responses of the experimental groups in the presence and absence of PEA were compared using χ^2 contingency analysis (Zar 1984). We assumed that all fish were acting independently. Fish which did not enter either trap were excluded from the analysis.

To determine whether immature fish with the same PEA-exposure history would respond differently to PEA than mature fish, 10 immature salmon from each exposure group were tagged, transported from the SP hatchery, released into the experimental arena on Dec. 13, and allowed to move freely until they entered a trap as described above.

II. Issaquah Creek Experiment

As another measure of PEA recognition, we tested whether salmon exposed to PEA at specific developmental stages and released as mature adults below the confluence of two unfamiliar streams would preferentially choose the stream fork scented with PEA. The study site for this experiment was the confluence of Issaquah Creek and its East Fork in Issaquah, Washington (Fig. 1). Issaquah Creek flows into Lake Sammamish in the Lake Washington watershed at a point 3.44 km downstream from the confluence of the two streams. On the main fork, the Washington State Department of Fisheries operates a weir and salmon hatchery 1.6 km upstream from the confluence with the East Fork. During this study, salmon migrating up the main fork were not allowed to pass the weir and entered a return pond at the hatchery. No traps or weirs were located on the East Fork of Issaquah Creek. The flow in the main channel was approximately 8-10 x greater than the East Fork, although both channels fluctuated considerably during this study.

We expected that in the absence of any familiar odors (i.e. PEA), most fish would either migrate up the larger (Main) fork or would not migrate upstream at all. On October 10, 1989, one week before releasing fish at the study site, we began metering PEA into the East Fork to establish a concentration of 10^{-6} - 10^{-7} M PEA in this channel. During the course of this study (Oct. 10-Nov 12), flow in the East Fork varied between 0.1 and 1.5 m^3/sec and the PEA drip was adjusted daily. On October 17, 100 mature fish (mean weight 1.19 kg) from each of the four PEA exposure groups (PEA-naive and alevin-, parr-, and smolt-exposure) were trucked from the SP hatchery and released into Issaquah Creek at a point 1.2 km downstream from the confluence of the two forks. Fish from

each treatment group were tagged with numbered, color-coded disc tags so that treatment group and individual identity could be determined from a distance. Fish migrating up the main fork of Issaquah Creek were monitored by recovering tagged fish in the hatchery return pond and weekly stream walks from the confluence to the hatchery ladder. Fish that migrated up the East Fork of Issaquah Creek were monitored three times per week by walking the stream from the confluence to a point 4.0 km upstream and recording experimental group, identification number and location of tagged fish. Both forks were monitored from October 19 until November 12 when heavy rains made surveys impossible and the PEA drip station was destroyed. Water temperatures in the forks were similar but varied between 7.0° and 11.5° C during the course of the study. The proportion of PEA-exposed fish choosing the PEA-scented East Fork vs. the main fork was compared to the proportion of PEA-naive choosing either fork using the Fisher Exact Test.

Continuous-exposure group

This group of salmon was established to determine the imprinting and homing ability of salmon exposed to a single water source throughout their life. It was impractical to continuously expose salmon to PEA so salmon were exposed to SP hatchery water. Eggs were collected at the UW hatchery on Nov. 30 1987, fertilized and initially incubated in dechlorinated Seattle city water. On Jan. 7 1988, eyed eggs were transferred to the SP hatchery and salmon were reared to maturity at this site. To determine the homing responses of these fish to SP water, we displaced them a short distance downstream and monitored the tendency of these fish to return to their hatchery. To test whether their return to the hatchery was guided by olfaction (rather than other site-specific features), we displaced salmon made anosmic by blocking the olfactory nares with wax.

On October 4, 1989, we anesthetized and tagged 145 mature continuous-exposure salmon with numbered tags. Thirty-five fish were made anosmic by injecting molten wax into both nares which rapidly solidified and blocked water access to the sensory epithelium. To control for handling stress, the remaining 100 fish were treated similarly by injecting warm water into the nares. After 48 h for recovery, the 100 control fish were transported and released from shore approximately 1.0 km north of the SP hatchery. To ensure that anosmic fish did not simply "follow" control fish back to the hatchery, they were held for an additional 24 h before transport and release. To ensure that the anosmia procedure did not affect survival, four anosmic and four control fish

were maintained at the hatchery. The trap at the SP hatchery was monitored daily for returning fish.

For comparison with the salmon reared exclusively in the hatchery, we also examined the homing tendency of anosmic and control salmon displaced downstream after completing their natural homing migration. Because the SP hatchery does not normally maintain a coho salmon run, these experiments were conducted at the UW hatchery with salmon that had returned to the hatchery after two years at sea. On October 26, 1988, anosmic and control groups of 20 salmon each were created as described above, transported approximately 5 km downstream and released from shore into the Lake Union Ship Canal. Each day the UW hatchery was surveyed for tags to determine the number of days to return for each group. As in the SP experiment, four anosmic and four control fish were maintained in the hatchery to assess the impact of the anosmia procedure on survival.

Determination of smolting status

Smolt transformation was determined by morphology and coloration changes and the ability of fish to maintain blood plasma sodium levels within 5% during a 48 hour exposure to sea water (SW). The hypo-osmoregulatory ability of the parr and smolt exposure groups was determined using a seawater challenge test (Clarke and Blackburn 1977) as modified by Sullivan (1986). Tests were conducted on May 15, 1988 for the parr groups and June 20, 1988 for the smolt groups. Tests were initiated by abruptly transferring fish from fresh water to aerated filtered sea water (27 ppt) maintained at the same temperature as the fresh water. After 48 h, blood was collected using heparanized capillary tubes and immediately centrifuged to isolate plasma. For comparison, plasma was also collected from control fish transferred to aerated fresh water (FW). Plasma osmolality was determined in duplicate for each sample using a vapor pressure osmometer and converted to plasma sodium (meq/l) using an empirically-derived regression equation for coho salmon (Sullivan 1986). Fish tested on May 15 (parr exposure group), were dark with distinct parr marks and were unable to maintain plasma sodium levels within 5% (FW exposure: 155.9 ± 0.8 meq/l plasma Na^+ , N=8; SW exposure: 179.7 ± 2.7 meq/l plasma Na^+ , N=12). However, fish tested on June 20, were elongate and silvery and were able to maintain plasma sodium levels after SW exposure (FW exposure: 152.9 ± 1.2 meq/l plasma Na^+ , N=10; SW exposure: 159.0 ± 1.7 meq/l plasma Na^+ , N=8).

Results

Migratory group

Of the 28,000 fish released as smolts from the SP hatchery, only 35 mature fish returned to the SP and UW hatcheries and these poor returns precluded statistical analysis. Fish exposed to UW water as alevins and control fish which never experienced UW hatchery water returned exclusively to the SP facility but a few salmon that experienced the UW as parr and smolts returned there (Table 2).

PEA group

Mature salmon tested in the experimental channel at Big Beef Creek without PEA present showed a preference for one arm (arm A) of the arena ($p < 0.05$ for all groups) and the proportion of fish choosing arm A was the same for all groups ($\chi^2=0.17$; $p < 0.99$) (Fig. 3, open bars). When we subsequently metered 10^{-7} M PEA into the less preferred arm (arm B), the choices of salmon in the PEA-naive ($p < 0.90$), alevin-exposure ($p < 0.99$) and parr-exposure ($p < 0.95$) groups were indistinguishable from their responses in the absence of PEA (Fig. 3). In contrast, mature salmon which had been exposed to PEA as smolts showed a greater tendency to enter arm B when PEA was present compared to responses in the absence of PEA ($\chi^2=3.97$; $p < 0.05$) (Fig. 3). Of the 440 mature fish tested, 83 (18.9%) chose neither arm of the channel during the experiment. Over the seven days of testing, none of the 40 immature salmon from the four PEA-exposure groups entered either arm of the arena. They remained primarily in the downstream section of the arena and appeared to be healthy and active.

The responses of mature PEA-exposed fish to PEA in Issaquah Creek were similar to the BBC results. The majority of fish from all treatment groups that migrated upstream were recovered in the Issaquah Creek hatchery (Table 1). However, the proportion of fish choosing the PEA-scented East Fork depended on their PEA exposure history. The proportion of mature salmon choosing the PEA-scented East Fork was the same for PEA-naive fish and fish exposed to PEA as alevins. On the other hand, salmon exposed to PEA as smolts demonstrated a greater tendency to migrate up the PEA-scented fork compared to controls ($p=0.018$). The parr exposure group showed a slight tendency to choose the PEA fork compared to controls ($p=0.12$). The majority of fish released below the stream confluence were not recovered and may have moved downstream and into Lake Sammamish, where several tagged fish were recovered. All but one fish

observed in the East Fork was sighted there on more than one occasion and no fish originally found in the East fork was subsequently captured at the hatchery on the main fork.

Continuous exposure group

Only 18% of the control salmon displaced 1.0 km from the Seward Park hatchery returned to that facility (Fig. 4). The time to return varied between 1 and 60 days. Eighteen days after release, two of the 100 fish released were recovered 18 km away in the UW homing pond. None of the 35 anosmic fish displaced from Seward Park returned to the SP hatchery. No difference in survival was apparent between anosmic (mean longevity = 21.25 days) and control (mean = 20.00 days) fish maintained at the hatchery during the course of this study.

Of the 20 control salmon released downstream from the UW hatchery, 70% returned to the UW hatchery pond within 5 days and 90% returned within 15 days (Fig. 4). The 10 % that did not return were captured in the commercial gillnet fishery in Lake Union. In contrast, none of the 20 anosmic salmon returned to the UW hatchery pond up to 60 days after release and none were reported captured in the fishery (Fig. 4). Interestingly, one anosmic fish was observed in the outflow of the UW return pond but never entered the pond. There was no difference in survival between treatment groups for fish maintained in hatchery raceways to determine if treatment-induced morbidity or mortality might have affected return rates (mean longevity= 14.75 days anosmic vs. 15.75 days control).

Discussion

Our PEA and site-specific exposure experiments indicated that the smolting stage is a particularly important period for olfactory imprinting in coho salmon reared in a hatchery. In two separate experiments, mature salmon that were exposed to 10^{-7} M PEA for only 10 days during smolting displayed an increased attraction to PEA-scented water 17 months later. Similarly, the return patterns of salmon exposed to the UW hatchery as smolts and released from the SP hatchery suggested that some of these fish had learned and were attracted to the UW site. Two of the five returning fish that were exposed to UW water as smolts were recovered at the UW hatchery. In contrast, 100% of the 14 fish in this experiment and 82 fish in a previous study (Brannon and Quinn 1990) that were

reared and released at SP hatchery without any UW exposure, bypassed the UW hatchery and returned to the SP hatchery. We have also found that PEA/smolt-exposed salmon form a long-term memory of PEA as evidenced by the increased sensitivity of their olfactory receptor neurons to this odorant (Nevitt et al. in press). Similar electrophysiology experiments with alevin- and parr-exposed fish were not performed.

Despite the evidence that smolting coho salmon learned olfactory cues for homing, there was no evidence for odor learning by alevins in these experiments. The evidence for imprinting by parr was weak. One of the seven returning fish that were exposed to UW water as parr returned to the UW hatchery as an adult. Similarly, there was a slightly increased tendency for salmon exposed to PEA as parr to migrate up the PEA-scented fork of Issaquah Creek. On the other hand, there was no indication of parr learning in the BBC study and in all cases the homing responses of fish exposed to odors as parr were weaker than smolt-exposed fish. These findings agree with previous studies implicating smolting as a critical period for olfactory imprinting. Scholz (1980) found that coho salmon were unable to imprint on artificial odorants prior to smolting and he proposed that olfactory imprinting in coho salmon is associated with elevated thyroid hormone levels that occur during smolting. Similarly, Morin et al. (1989a) found that Atlantic salmon had an optimal period for long-term olfactory learning 21-28 days after the onset of smolting, coinciding with peak levels of thyroid activity (Morin et al. 1989b).

The absence of any pre-smolt learning contrasted with Courtenay's (1989) findings that coho salmon exposed to morpholine, another artificial odorant, as pre-hatch embryos, alevins or fry responded behaviorally to this odor when tested 2 to 4 months later. Furthermore, Courtenay's fish maintained an olfactory memory for morpholine more than one year after exposure, as indicated by heart rate reductions in the presence of this odorant. The differences between our results may reflect the different developmental status of the salmon tested for odor recognition. Hasler and Scholz (1983) reported that only mature coho salmon responded behaviorally to imprinted odorants. Electrophysiology and heart rate conditioning experiments also indicated that olfactory sensitivity to imprinted odorants is greatest during maturation (Cooper and Hasler 1973; Hasler and Scholz 1983).

We conducted odor recognition experiments with mature adults while the fish in Courtenay's (1989) study were immature and the long-term odor memories he demonstrated may not have been associated with reproductive homing. Pre-smolts do

learn odors associated with specific habitats (e.g. lake vs. river water) (Brannon 1972; Bodznick 1978; Halvorsen and Stabell 1990) and odors from other fishes (e.g. Quinn and Hara 1986; Courtenay 1989) but it is not known whether such odor memories are important for homing. In our BBC experiments, immature salmon did not respond to PEA-scented water regardless of PEA exposure history. Whether this reflects an inability to recognize PEA or lack of motivation to respond is not clear but it does indicate that mature and immature salmon respond differently to odors learned as juveniles. It is important to note, however, that homing and maturation are not inextricably linked. Many populations of salmon migrate back to their river of origin months before maturing (e.g. spring chinook and summer steelhead). In these races, fish may home hundreds of km upriver to general vicinity of their natal stream, then hold in the mainstem river for several months before resuming their homing to the spawning site (e.g., Berman and Quinn 1991).

One explanation for why we observed no pre-smolt learning may lie in the methods we and others have used to assay odor recognition and attraction. As part of the sequential imprinting hypothesis, Harden Jones (1968) proposed that during their homing migration, salmon retrace their seaward migration by first seeking the last odor they experienced before going to sea, then the next-to-last odor and so on in sequence until they arrive back at the natal site. If this is correct, the salmon in our study may have imprinted prior to smolting but did not respond to these odors because they didn't first experience the odors they learned as smolts. This explanation is consistent with our PEA experiments and data from displacement experiments on the Columbia River in which salmon released upriver from their rearing site migrated past the rearing site as adults and returned to the release site (Slatick et. al 1988; Pascual 1993). In one Columbia River experiment, however, juvenile coho salmon released upstream of their hatchery returned as adults to their rearing hatchery, not the release site (Johnson et al. 1990). In our displacement experiments, 3 of the 35 fish returning to Lake Washington were recovered at the UW hatchery but it is uncertain whether these fish entered the UW hatchery when they first encountered UW water or whether they first returned to the vicinity of SP and then began searching for UW water.

Another factor which may have contributed to the lack of any observed pre-smolt learning is the different fresh water environments experienced by hatchery-reared and wild fish. Nishioka et al. (1985) hypothesized that the stable rearing conditions in most hatcheries (e.g., constant temperature, flow rate, water source) may provide insufficient

environmental stimuli for optimal thyroid development. Under such conditions, hatchery fish may only experience an endogenously controlled smolting-related increase in thyroxine levels. In the wild, salmon experience various environmental changes which can elicit multiple thyroxine surges throughout the period of freshwater rearing (Nishioka et al. 1985). Exposure to novel water, changing lunar cycles, increased water velocity and temperature fluctuations have all been linked to increased thyroid activity (Grau et al. 1981; Dickhoff et al. 1982; Iwamoto 1982; Youngson and Simpson 1984; Nishioka et al. 1985). If thyroid hormones are important for olfactory imprinting, this might explain the apparent inconsistencies between experimental evidence obtained with hatchery-reared fish which points to smolting as a critical period for imprinting and studies with wild fish which indicate that salmon must also learn at other times (Chapter III).

While our results clearly indicated that smolts were able to learn odors for homing, the imprinting and homing ability of these fish was unremarkable compared to naturally migrating UW fish. Coded wire tag data indicated that UW coho salmon home with extraordinarily precision when released from the hatchery as smolts (Quinn et al. 1989). In contrast, 72% of the 400 fish released 1.5 km below the PEA drip site in the Issaquah Creek study were never recovered and may have emigrated from the river as might be expected in the absence of any learned odors (Johnsen and Hasler 1980). Furthermore, the majority of upstream migrants from all treatment groups chose the unscented but larger Main Fork vs. the PEA-scented East Fork. These results agree with Wisby and Hasler's (1954) results from the same site; in the absence of familiar odors salmon tended to enter the fork with the greater flow. Similarly, in the BBC experiment, smolt-exposed salmon were more likely to enter the PEA-scented arm of the arena than PEA-naive fish but 48.3 % of these fish chose the non-scented arm.

We were intrigued by the poor homing abilities of salmon exposed continuously to SP water. Only 18 of the 100 salmon displaced just 1.0 km were recovered at the SP hatchery, two fish were recovered at the UW hatchery which they never experienced and the time to return to SP was as high as 60 days. In contrast, 90% of the salmon that were released as smolts and experienced seaward and homing migrations returned to their home hatchery within 15 days. As expected, none of the anosmic fish returned to their home hatchery, confirming that the olfactory sense is required for salmon homing (Wisby and Hasler 1954). The differences between the return rates of salmon displaced from SP and UW were not due to any inherent problems with the SP hatchery as a return site. One

hundred per cent of the coho salmon reared and released from the SP hatchery as smolts were recovered there at maturity (Brannon and Quinn 1990; this study).

The impaired homing ability of fish retained in the hatchery after smolting (i.e., PEA-exposure and continuous exposure groups) vs. fish released as smolts suggests that exposure to odors during smolting alone may be necessary but not sufficient for optimum imprinting. There may be a sensitive window during which salmon must migrate for full imprinting to occur. Salmon released from hatcheries as smolts show much higher levels of homing than those released after the smolt period (Chinook salmon: Unwin and Quinn 1993; Pascual 1993; Atlantic salmon: Hansen and Jonsson 1991). Furthermore, displacement experiments in which smolts are trucked downstream for release indicate that while some fish return to their natal site the majority return as adults to the area where they began their downstream migration (Slatick et al. 1988; Quinn et al. 1989).

How might migration be involved in imprinting? During migrations, swimming rates often increase and salmon move into new environments of differing water chemistry, temperatures and flow rates. All these factors can stimulate T4 production (Dickhoff et al. 1982, Nishioka et al. 1985) and migrating salmon may therefore experience elevated T4 levels which may facilitate olfactory imprinting. This may also explain why wild salmon apparently learn odors as fry (perhaps when they migrate from these sites) but salmon held in hatcheries show less evidence of such learning.

In summary, our results confirm the importance of smolting as a sensitive period for olfactory imprinting but suggest that exposure to site-specific water alone is not sufficient for complete imprinting. Furthermore, there was little evidence for pre-smolt learning of homing cues by hatchery-reared fish. Since salmon in the wild often learn home site odors prior to smolting, the lack of any observed early learning in our study may be due to hatchery rearing conditions, lack of migration, or population-specific learning patterns. Finally, our results suggest that while migration is not absolutely required for imprinting, the combination of stimuli associated with migration and physiological changes involved in smolting may be important for optimal imprinting and homing.

FIGURE 1. Map of the Lake Washington watershed showing the location of the University of Washington (UW) and Seward Park (SP) hatcheries and release locations for displacement experiments. Inset shows the Issaquah Creek study site.

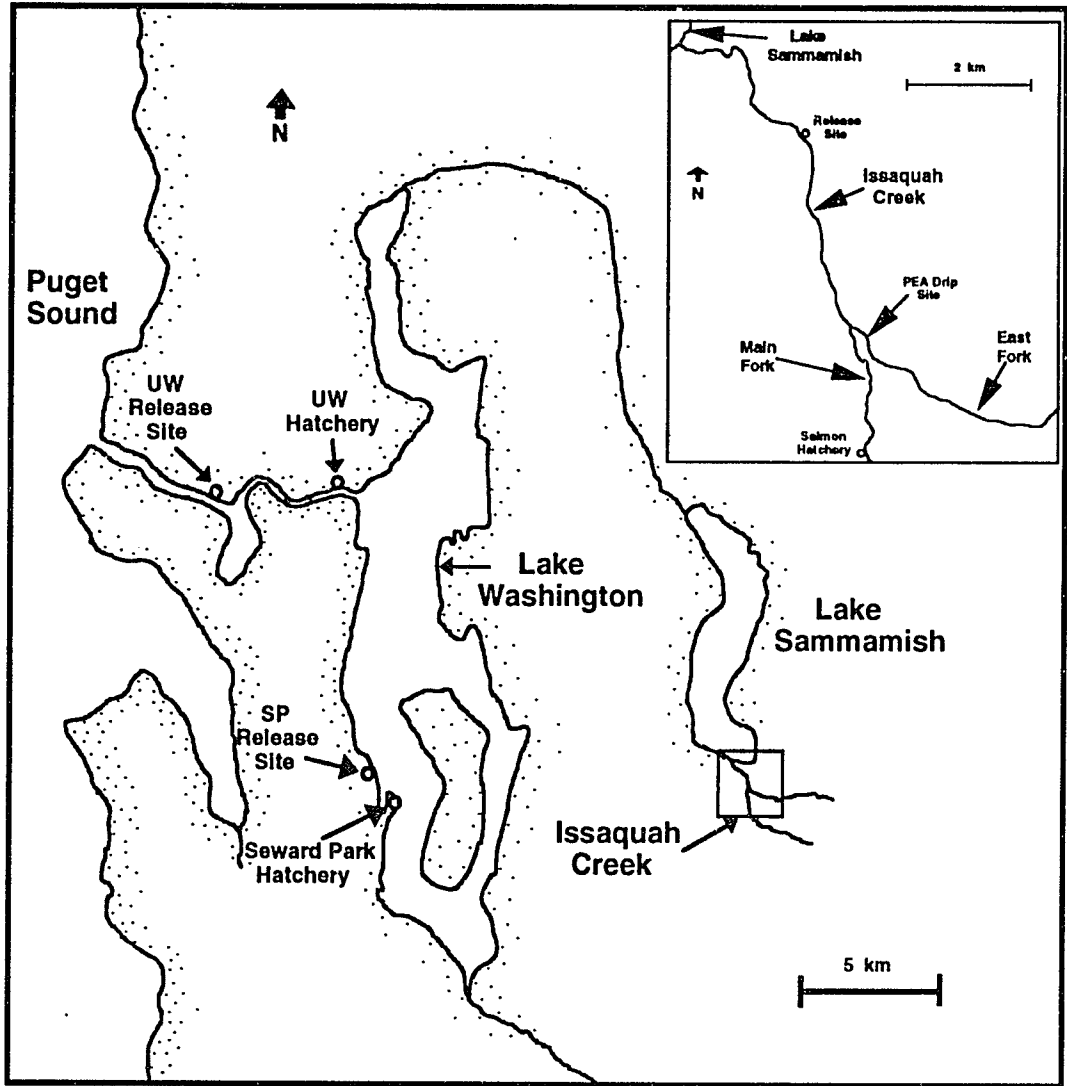
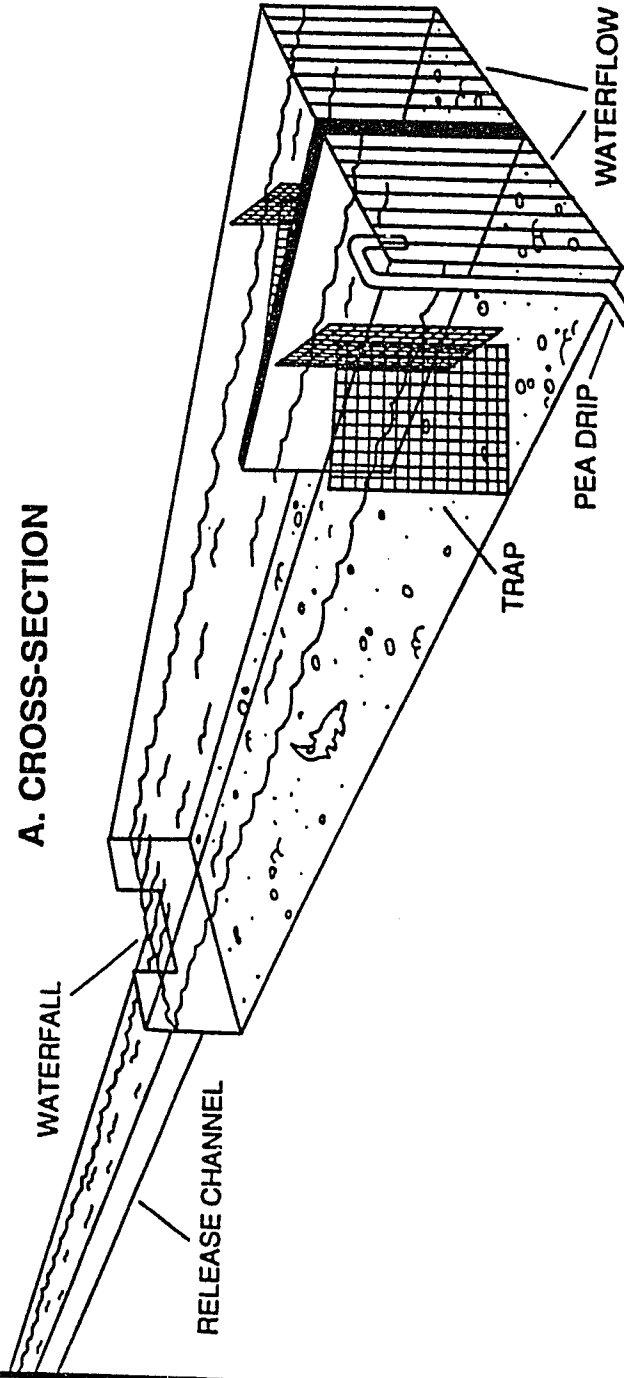


FIGURE 2. Two-choice arena for testing behavioral recognition of imprinted odorants located at the University of Washington's Big Beef Creek research station, Kitsap Peninsula, Washington.

A. CROSS-SECTION



B. TOP VIEW

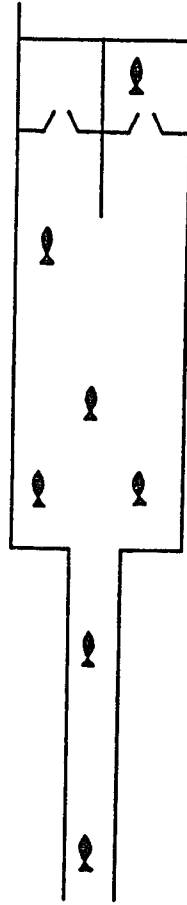


FIGURE 3. Behavioral responses of mature coho salmon to PEA in the BBC two-choice arena. Salmon were exposed to PEA at the developmental stage indicated or never experienced PEA (control). Open bars show the proportion of salmon choosing arm B in the absence of added PEA. Shaded bars show the proportion of salmon choosing arm B in the presence of PEA metered into arm B. Numbers above bars indicate the total number of fish choosing either arm A or B.

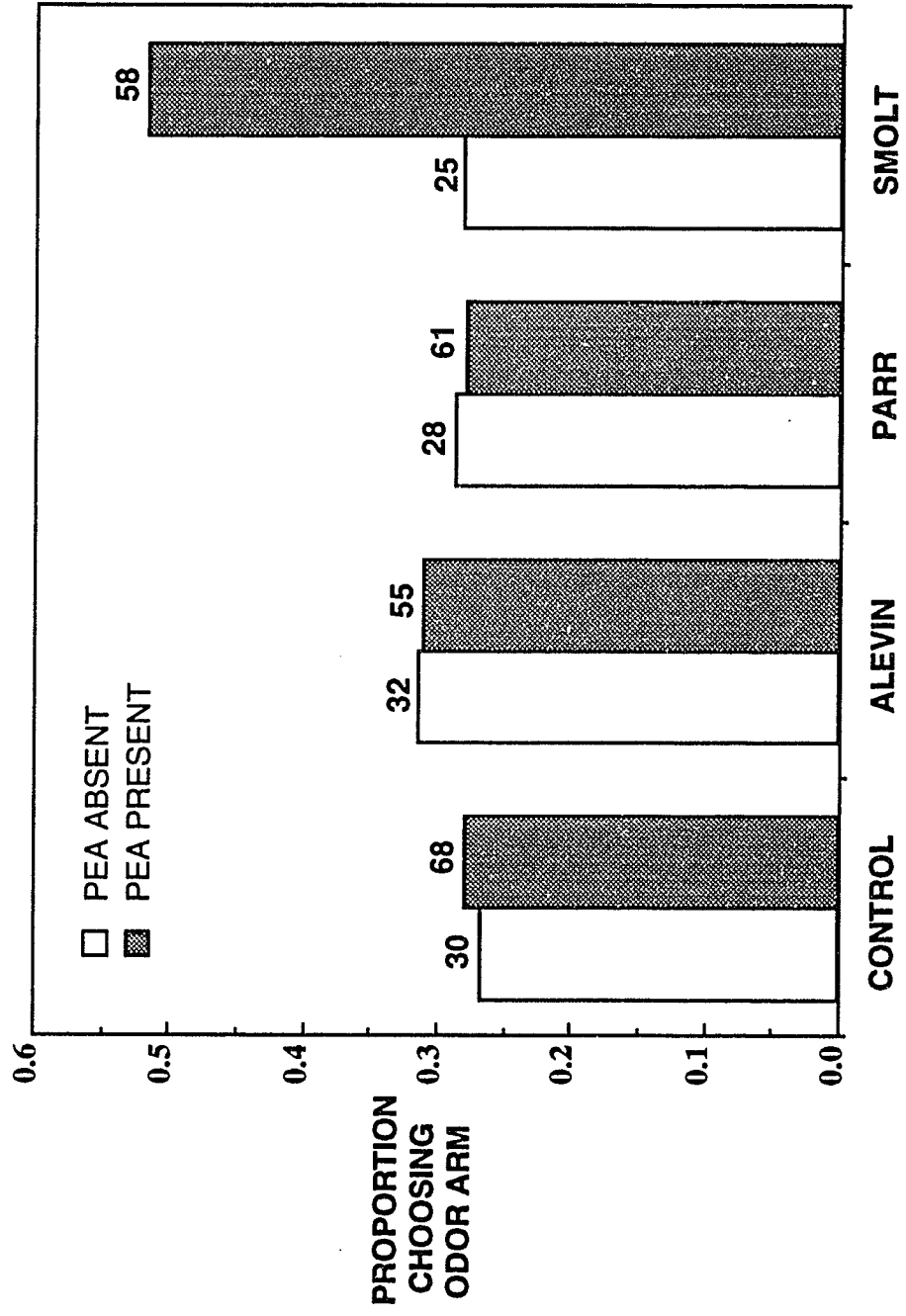


FIGURE 4. Cumulative frequency plot of the number days for anosmic and control salmon to return to their home hatchery after release downstream from the Seward Park (SP) and University of Washington (UW) hatcheries. Prior to displacement, SP fish were exposed to SP water throughout their life while UW fish were released from their hatchery as smolts.

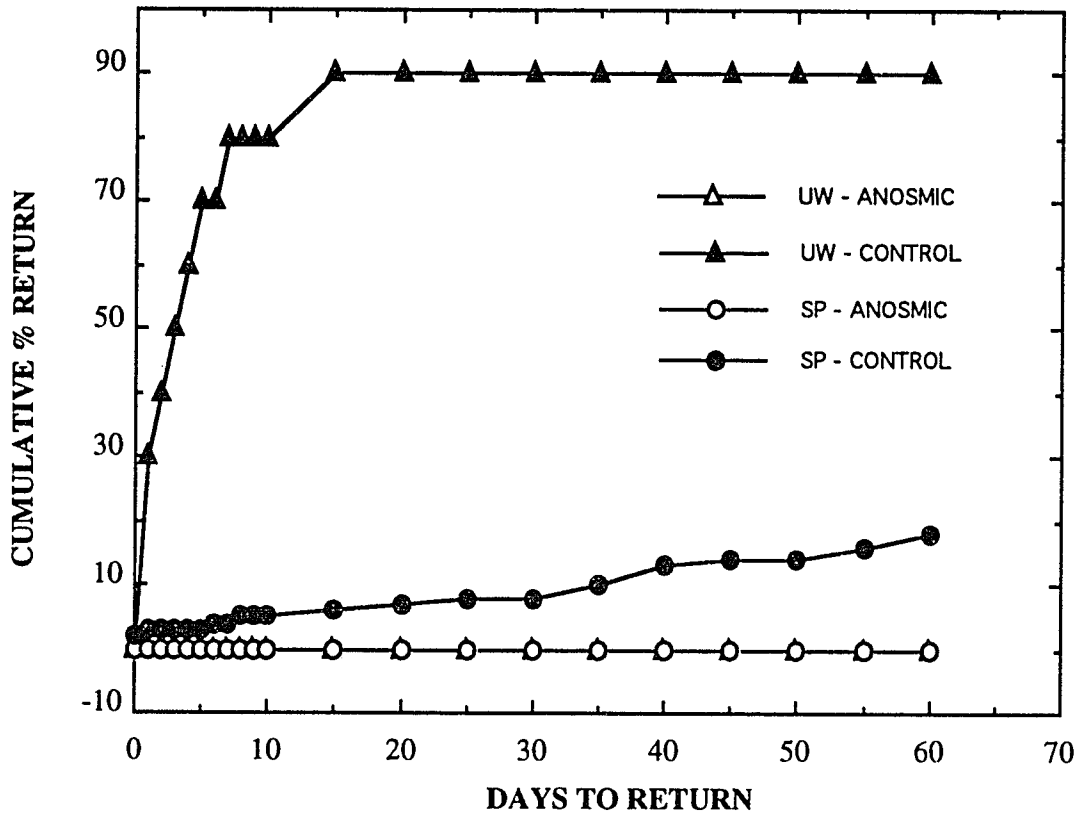


TABLE 1. Numbers of adult coho salmon recovered in either the PEA-scented East Fork or the Main fork (control) of Issaquah Creek after release 1.2 km below the confluence of the two streams. Salmon were exposed to 10⁻⁷ M PEA at the developmental stage indicated. Control fish never experienced PEA.

Recovery site	Treatment			
	<u>Control</u>	<u>Alevin</u>	<u>Parr</u>	<u>Smolt</u>
Main Fork (control)	25	25	24	24
East Fork (PEA-scented)	1	1	5	9

Table 2. Patterns of homing displayed by adult coho salmon reared and released at the Seward Park hatchery except for brief exposures to UW hatchery water at the developmental stage indicated. Control fish never experienced UW water.

Recovery site	Treatment			
	<u>Control</u>	<u>Alevin</u>	<u>Parr</u>	<u>Smolt</u>
Seward Park	14	9	6	3
UW	0	0	1	2
Commercial and sport fishery	13	13	7	0

Chapter II

Interactions Between Novel Water, Thyroxine and Olfactory Imprinting in coho salmon

Introduction

Pacific salmon (*Oncorhynchus* spp.) migrate from oceanic feeding grounds to their river of origin to spawn. The final freshwater phase of this migration is primarily governed by olfactory discrimination of home stream waters learned by juveniles prior to their seaward migration (Hasler and Scholz 1983). This was demonstrated in a series of classic experiments by Hasler and his colleagues that showed that juvenile coho salmon (*O. kisutch*, Walbaum) exposed to synthetic chemicals, such as morpholine or phenylethyl alcohol (PEA), could subsequently be attracted into unfamiliar streams scented with these chemicals during their spawning migration (Cooper et al. 1976; Scholz et al. 1976). Olfactory learning, or imprinting, of home stream specific odors is thought to occur during a single sensitive period associated with smolting; the physiological, morphological and behavioral transformation that readies juvenile salmon for ocean life (Hoar 1976; Folmar and Dickhoff 1980). Many of these adaptations are associated with surges in the hormone thyroxine (T4) that occur during smolting (Dickhoff et al. 1978; Dickhoff and Sullivan 1987) and these high T4 levels are thought to be involved in olfactory imprinting (Hasler and Scholz 1983; Morin et al. 1989a, b). This link between thyroid hormones, smolting and imprinting is consistent with displacement experiments indicating that salmon reared at one site but released at second site prior to seaward migration generally return as adults to the release site, not to their rearing site (Donaldson and Allen 1959; Jensen and Duncan 1971).

While experimental evidence with hatchery-reared fish suggests that the smolting period is critical for imprinting, the complex movement patterns of juvenile salmon in the wild indicate that olfactory imprinting to natal streams must also occur prior to smolting. For example, most sockeye salmon (*O. nerka*, Walbaum) emerge from redds in their natal streams and immediately migrate downstream to rearing lakes where they live for 1 or 2 years before smolting and migrating to sea. However, during their homing migration, adults migrate past the outlet streams and lakes experienced as smolts and return to their natal stream to spawn. Coho and chinook (*O. tshawytscha*, Walbaum) salmon also may

move extensively in freshwater prior to seaward migration (e.g. Peterson 1982; Murray and Rosenau 1989) yet home to their site of incubation. Such inferential evidence for pre-smolt imprinting is supported by Courtenay's (1989) findings that juvenile coho salmon can learn odors they experience shortly after or even before hatching and lends support to the hypothesis that imprinting can occur at more than one place and time.

An explanation for the paradox of experimental evidence of a critical link between imprinting and high plasma T4 levels during smolting and the fact that many salmonids must learn at earlier stages may lie in recent endocrinological studies of juvenile salmon. T4 surges occur not only during smolting but may also occur during hatching and as parr (Dickhoff and Sullivan 1987). Moreover, exposure to a novel water source can cause a transient increase in plasma T4 levels in smolts (Dickhoff et al. 1982; Grau et al. 1985; Lin et al. 1985; Nishioka et al. 1985; Hoffnagle and Fivizzani 1990). If thyroid hormone levels are associated with olfactory imprinting and novel water induces T4 surges, then juvenile salmon migrating through a watershed might learn olfactory waypoints each time they experience novel water (e.g., at the confluences of rivers) which they retrace years later as homing adults. Such a scenario is consistent with sequential imprinting, proposed by Harden Jones (1968) and Brannon (1982).

We have hypothesized that as a juvenile salmon migrates within a watershed, exposure to novel odors induces a transient increase in plasma T4 levels which is associated with olfactory learning (Quinn 1990; Quinn and Dittman 1990). If so, we would expect that these T4 surges can occur prior to smolting and in response to imprintable odorants. To test this hypothesis, we examined whether exposure to the artificial odorant PEA or chemically distinct novel water at different developmental stages could elicit transient T4 surges. In 1990, we chose 10^{-7} M PEA as a test odorant because brief exposure to PEA at this concentration is sufficient for olfactory learning and coho salmon will retain long-term odor memories for PEA that are demonstrated by both electrophysiological and behavioral measures (Hasler and Scholz 1983; Nevitt 1990). In 1991, we repeated experiments with PEA and also examined whether more dramatic changes in water would affect T4 levels in coho salmon. Finally, we examined the seasonal changes in plasma T4 levels in subyearling vs. yearling UW coho salmon during smolting.

Methods

Experimental Animals The salmon used for this study were from the University of Washington's (UW) coho salmon run. Except where noted, fish were subyearling salmon reared as part of the normal hatchery production at the UW hatchery. The UW coho salmon are reared under an accelerated feeding and temperature regime and natural photoperiod which allows them to undergo smolting in the first spring after hatching (Brannon et al. 1982). UW coho salmon typically spawn in late October and November and are released as smolts in late spring the following year. Prior to novel water testing, salmon were reared exclusively in hatchery water (UW) which is pumped from the Lake Washington Ship Canal.

Experimental Procedure 1990 During 1990, novel water experiments were conducted on three dates representing three distinct developmental stages: parr group (March 30-April 3: 13.5-14.0 °C); smolt group (June 2-5: 14.2-15.2° C); post-smolt group (August 7-10: 16.5-17.0° C). Three weeks prior to testing, salmon were moved from communal rearing troughs to two experimental troughs also containing UW water. Just prior to transfer and 48 hours after transfer, plasma was collected as described below to examine the effects of transfer on T4 levels. In 1990, we tested whether exposure to the artificial odorant, PEA, would elicit increases in plasma T4 levels. Just prior to PEA exposure, fish were randomly collected from each trough and killed with a blow to the head. Blood was collected in heparanized capillary tubes after severing the caudal peduncle. Blood from three groups of 10 fish was pooled for the parr group (mean weight= 4.05 g), 3 groups of 2 fish for the smolt group (mean wt.= 20.08 g), and 4 individual fish for the post-smolt group (mean wt.= 37.48 g) for each experimental treatment and time point. Blood was centrifuged at 10,000 x g for 3 min. and plasma was separated and stored at -20° C for T4 analysis. Plasma T4 concentrations were determined in duplicate using a specific radioimmunoassay as described (Dickhoff et al. 1978). To initiate testing, PEA was metered into one trough to a final concentration of 10^{-7} M while the other trough remained as a control. Plasma was subsequently collected from both groups, as described, at 2, 24, 48, and 72 hours after initiating the PEA drip. Flow rates in both troughs were maintained at approximately 16 L/min throughout the testing. During PEA exposure, salmon were fed twice daily to satiation.

Experimental Procedure 1991 In 1991, we tested whether exposure to PEA, 10% novel water, and 100% novel water would elicit increases in plasma T4 concentrations. The novel water used in these experiments was dechlorinated Seattle city water (SC) which is drawn from the Tolt River reservoir located outside the Lake Washington watershed and is distinguishable from UW water by coho salmon (Quinn et al. 1983). Novel water experiments were conducted on June 3-6 with salmon at two distinct developmental stages. The smolt group (mean wt.= 14.23 g) consisted of fish from eggs fertilized in early November 1990 and reared under the normal accelerated feeding regime. The parr group (mean wt.= 3.65 g) consisted of offspring from late (early December) spawning fish which were fed a more limited diet. These fish did not undergo smolting as subyearlings. Three weeks prior to testing, 225 smolts and 500 parr were transferred to divided sections of each of 4 experimental troughs containing UW water. Just prior to transfer and 48 hours after transfer, plasma was collected to examine the effects of transfer on T4 levels. Immediately before novel water exposure (t=0 h), plasma was collected from 4 groups of 5 parr and 10 individual smolts randomly collected from each trough. This collection procedure was followed at all subsequent time points. To initiate testing, PEA was metered into trough 1 to a final concentration of 10^{-7} M. The 10% novel water group was initiated by supplementing UW hatchery water with 10% SC water in trough 2. The 100% novel water treatment was initiated by switching trough 3 from UW water to only SC water. Trough 4 was maintained as a control (no water change). In all troughs, the total flow rate was maintained at pre-treatment levels (12.5-13 L/min) by adjusting flow rates of the two component waters. Plasma was collected from all parr and smolt treatment groups as described above, at 2, 24, 48, 72, and 168 hours after initiating the novel water exposure. Water temperature during the experiment varied from 14.6° to 15.6° C but UW and SC water differed by less than 1° C during the course of the experiment. During novel water exposure, salmon were fed twice daily to satiation.

Determination of Smolting Status Smolt transformation was determined by morphology and coloration changes and abilities of fish to maintain plasma sodium levels during a 24 hour exposure to seawater. During 1990 and 1991, the hypo-osmoregulatory ability of all experimental groups was determined using a seawater challenge test (Clarke and Blackburn 1977) as modified by Sullivan (1986). The test measured the ability of fish to regulate their blood plasma osmolality after transfer from fresh water to seawater. All tests

were conducted at the same time as novel water/thyroxine testing. Tests were initiated by abruptly transferring either 50 fish (parr in 1990 and 1991) or 25 fish (smolts in 1990 and 1991 and post-smolts in 1990) from fresh water to aerated filtered seawater maintained at the same temperature as fresh water. Water temperature varied slightly during the year (e.g. 14.0° C parr 1990, 15.0° C smolt 1990, 16.5° C post-smolt 1990). After 24 hours in seawater, the per cent mortality was determined and blood plasma from surviving fish was collected as described earlier. For comparison, plasma was also collected from control fish transferred to aerated fresh water. Plasma osmolality was determined in triplicate for each sample using a vapor pressure osmometer. Estimates of plasma sodium (meq/l) were derived from osmolality measurements using an empirically-derived regression equation (Sullivan 1986).

Results

The introduction of novel water caused no changes in mean plasma thyroxine levels in subyearling coho salmon within 72 hours of exposure at any developmental stage tested (Figures 5 and 6). In 1990, exposure to 10^{-7} M PEA did not elevate plasma T4 levels in comparison to control fish during the parr, smolt, or post-smolt stage (Figure 5). In both control and novel water-treated fish, only slight seasonal changes in mean plasma T4 levels were observed during 1990. Plasma T4 rose from an initial level of 4.44 ng/ml on March 10-12 (parr group) to 6.26 ng/ml on June 2-5 (smolt group) and then declined to 4.39 ng/ml T4 during the final August 7-10 test period (post-smolt) (Figure 7). These results with underyearling smolts differed from previous studies with yearling UW coho smolts (Sullivan 1986; Sullivan et al. 1987) which displayed a large and more typical 1-2 month smolting-associated increase in plasma T4 levels (Figure 7).

Morphology, coloration, and hypo-osmoregulatory capabilities all indicated that the fish did undergo smolt transformation during this period. On April 1, 1990 (parr group), salmon were dark with very distinct parr marks. After 24 hours in seawater (SW), 30 % of these fish died and the plasma sodium levels in surviving fish were significantly higher than fresh water (FW) controls (SW: 231.80 ± 5.21 meq. $\text{Na}^+/\text{l} \pm \text{S.E.M}$ vs. FW: 153.82 ± 2.28 meq/l) indicating that they had not smolted (Clarke and Blackburn 1977). On June 5 (smolt group), however, salmon were elongate, silvery, suffered no mortality after 24 hours in SW and were able to maintain their plasma sodium within 5-10% of the levels in FW (SW: 155.54 ± 1.78 vs FW: 146.05 ± 0.91 meq/l), indicating that they had

successfully smolted. Post-smolts (Aug. 10) maintained their silvery coloration, suffered only 5% mortality in SW and were intermediate in their hypo-osmoregulatory abilities (SW: 168.74 ± 16.5 meq/l vs 151.68 ± 1.55 meq/l).

In 1991, exposure to several different novel water regimes failed to elevate plasma T4 levels in parr or smolts compared to control fish during a 72 hour exposure (Figure 6). As in 1990, exposure to 10^{-7} M PEA elicited no change in plasma T4 levels in parr or smolts. Furthermore, exposure of parr and smolts to either 10% or 100% Seattle city water caused no significant changes in plasma T4 concentrations. The basal level of T4 in smolts (4.43 ± 0.26 ng/ml) was only slightly higher than the parr group (3.06 ± 0.12 ng/ml). As in 1990, morphology, color, and osmoregulatory capabilities indicated that fish designated "parr" had not smolted (SW challenge; SW: 200.31 ± 3.20 vs. FW: 150.22 ± 2.81) whereas the "smolt" group had undergone smolting (SW: 168.74 ± 1.74 meq/l vs. FW: 155.54 ± 1.78 meq/l). In 1991, plasma T4 levels were also examined 7 days after novel water exposure was initiated because this day coincided with the new moon and Nishioka et al. (1985) found that novel water surges only occurred during the new moon. For both parr and smolts, neither control fish nor the three novel water experimental groups demonstrated a statistically significant rise in plasma T4 on this date. One fish in the smolt/10% SC group did have dramatically higher T4 levels (19.4 ng/ml) on this date.

In both 1990 and 1991, transferring salmon from rearing tanks to experimental troughs containing the same UW hatchery water had no effect on T4 levels at any stage tested (Table 3).

Discussion

Exposure to novel water or modified water chemistry (PEA) did not influence plasma thyroxine levels in subyearling coho salmon at any developmental stage tested. In both years, introduction of PEA at a concentration which is learned and remembered by UW coho smolts (Nevitt et al. in press) had no significant effect on plasma T4 levels in either smolts or non-smolts. These results differed from previous reports indicating that the addition of low concentrations of chemicals, such as amino acids and/or salts, to the rearing water elicited T4 surges (Specker and Shreck 1984; Nishioka et al. 1985). While the concentration of PEA we used was sufficient for learning, previous novel water

studies added concentrations of chemicals at least two orders of magnitude higher. To test whether 10^{-7} M PEA may have been too subtle a change in water chemistry to elicit a T4 response, we repeated this experiment with more dramatic water changes. Introduction of 10% SC or 100% SC water had no effect on plasma T4 levels in either smolts or pre-smolts compared to fish maintained in UW water. While the lack of T4 changes in pre-smolts might indicate that coho salmon do not demonstrate novel water T4 surges prior to smolting (Lin et al. 1985; Grau et al. 1985), we were surprised that exposure to completely novel water also did not affect plasma T4 levels in smolts as has been reported with yearling coho (Dickhoff et al. 1982; Lin et al. 1985; Nishioka et al. 1985; Grau et al. 1985) and chinook salmon (Hoffnagle and Fivizzani 1990).

It is possible we did not observe novel water induced T4 surges because of the sampling paradigm used. In previous studies, novel water exposure elicited a rapid and transient rise in plasma T4 levels in yearling coho smolts (Dickhoff et al. 1982; Grau et al. 1985; Lin et al. 1985; McCormick and Saunders 1990). For example, Dickhoff et al. (1982) found that transferring yearling coho smolts to a new water source caused a two-fold increase in plasma T4 concentrations within 24 hours and T4 returned to control levels within 72 h of exposure. Based on this previous work, we limited our T4 sampling to a 72 h period following novel water exposure except for one 7 day time point in 1991. However, Hoffnagle and Fivizzani (1990) found that chinook salmon exposed to novel water did not demonstrate differences in plasma T4 levels until 10 days after exposure. Furthermore, Nishioka et al. (1985) found that in some cases coho salmon smolts only experienced novel water T4 surges during the new moon. An association between phase of the lunar cycle and thyroid activity in salmon is well documented (Grau et al. 1981, 1982; Yamauchi et al. 1984). In our study none of the novel water exposures coincided with the new moon and if novel water T4 surges occurred only during these period we might not have observed them. However, if T4 surges and imprinting are to occur as salmon migrate downstream and experience river confluences, they must not lag exposure by 10 days or only take place on selected periods of the lunar cycle but must rapidly follow novel water exposure.

In addition to phase of the lunar cycles, a number of environmental cues have been linked to increased thyroid activity including temperature changes (Iwamoto 1982; Lin et al. 1985), photoperiod (Hoar 1976; Iwamoto 1982), water flow rates (Youngson and Simpson 1984; Lin et al. 1985) and food intake (McCormick and Saunders 1990). All of these factors can apparently contribute to T4 increases both independently and in

conjunction with smolting-associated T4 surges. Nishioka et al. (1985) hypothesized that in some instances, the stable conditions (e.g., constant temperature, flow rate, water source, ration) which allow for increased survival in the hatchery may provide insufficient environmental stimuli to fully stimulate thyroid function. In most hatcheries, smolting-associated T4 surges occur (Dickhoff et al. 1978, 1982; Nishikawa et al. 1979; Folmar and Dickhoff 1981) but in some instances hatchery-reared salmon experience little or no smolting-associated T4 surge (Youngson and Simpson 1984; Nishioka et al. 1985; Lin et al. 1988). Similarly, we observed no T4 surge during smolting in our experimental fish, although it appears the underyearling status of UW smolts may be the primary reason (see below).

The lack of any smolting-associated T4 surge may provide some explanation for the absence of any apparent novel water-induced T4 surge in our experiments. The novel water phenomenon may be primarily observed when the thyroid system is previously "primed" either through the normal smolting induced T4 surge (Grau et al. 1985; Lin et al. 1985; McCormick and Saunders 1990) or by environmental cues (e.g. lunar phase, Nishioka et al. 1985). Since the underyearling smolts in our study never experienced a T4 surge during smolting, they may not have been sufficiently "primed" to respond to novel water exposure.

In the wild, salmon may experience various environmental changes which may elicit multiple thyroxine surges throughout the period of freshwater rearing (Nishioka et al. 1985). For example, wild fish may periodically experience dramatic changes in water velocity or temperature due to rainfall and snowmelt. Increased water velocity forces fish to swim more actively to maintain their position in the stream and the increased swimming has been shown to cause T4 levels to rise (Dickhoff et al. 1982; Youngson and Simpson 1984). Fluctuations in water temperature have also been linked to increased T4 levels (Iwamoto 1982). On the other hand, hatchery fish are generally maintained in a stable environment and may only demonstrate an endogenously controlled, smolting-related increase in T4 levels. If thyroid hormone signalling is important for olfactory imprinting, this might help explain the apparent contradiction between experimental evidence with hatchery reared fish, which points to smolting as critical period for imprinting, and the natural history of wild salmon, that indicates learning must occur at other times. It may be that imprinting and homing experiments with hatchery fish are compromised by the unchanging rearing conditions which foster minimal thyroid development beyond the smolt associated T4 surge. In the wild, however, constantly changing environmental

stimuli can elicit multiple thyroxine surges and prime the thyroid system for novel water exposure induced T4 increases which may facilitate learning at other times.

While insufficient environmental stimuli may be important in some hatcheries, the absence of a smolting-associated T4 surge in UW coho salmon in 1990 and 1991 was apparently due to the underyearling status of UW coho smolts. Typically, coho salmon smolt and migrate to sea during their second spring after 1.5 or 2.5 years in fresh water. At the UW hatchery, however, coho salmon are reared on an accelerated temperature and feeding regime which causes them to smolt in their first spring (i.e. underyearling smolts). Under these rearing conditions all morphological, behavioral and physiological measures indicated that these fish successfully smolted but they experienced no apparent smolting associated T4 surge (Figure 7). Sullivan (1986) also found that UW underyearling smolts demonstrated no rise in T4 levels during smolting although basal plasma T4 levels were significantly higher than we observed. On the other hand, if UW coho salmon are reared under a more natural feeding and temperature regime, they smolt as yearlings and demonstrate a normal 1-2 month rise in plasma T4 levels in late spring (Figure 7; Sullivan 1986; Sullivan et al. 1987).

The ability of hatchery-reared salmon to migrate and return accurately to their site of origin without experiencing a smolting associated T4 peak has been previously reported (Lin et al. 1988; Nishioka et al. 1989). The lack of any T4 surge in UW underyearling smolts is particularly interesting, however, in light of the considerable research directed at the imprinting and homing abilities of UW coho salmon (e.g. Donaldson and Allen 1957; Quinn et al. 1989; Brannon and Quinn 1990). Exposure of UW underyearling coho salmon to specific waters during smolting consistently results in olfactory learning despite the relatively low and unchanging plasma T4 levels during this period. For example, recoveries of coded wire tagged salmon indicate that UW underyearling coho smolts are able to successfully smolt, imprint, migrate to sea and return as adults to the UW hatchery with remarkable precision (e.g. Quinn et al. 1989). Furthermore, UW underyearling smolts are also able to learn other water sources as home. UW smolts reared and released at a different hatchery in the Lake Washington watershed migrated past the UW hatchery and homed to their release site (Quinn et al. 1989). This imprinting process occurred primarily during the smolt stage for UW coho salmon. Salmon reared initially at the UW and then transferred at the smolt stage to a second facility for subsequent rearing and release, return to their release site not the UW hatchery (Chapter I). Furthermore, UW underyearling salmon which are exposed to 10^{-7} M PEA for as little as 10 days during

smolting retain long term odor memories of this chemical as evidenced by both electrophysiological and behavioral responses to PEA by adults (Nevitt et al. in press). Taken together, these findings suggest that high levels or surges of plasma T4 levels are not required for olfactory imprinting and cast doubt on a critical link between T4 and imprinting.

While T4 may not be critical for imprinting, it is possible that thyroid hormone signalling may be important given the considerable evidence suggesting a link between smolting, thyroid function, and olfactory learning (Hasler and Scholz 1983; Morin et al. 1989a, b). Thyroid system function is regulated at many levels and plasma T4 concentration is only one measure of thyroid activity. In salmon, T4 is converted in the periphery to the more active metabolite, triiodothyronine (T3), which binds and acts on nuclear T3 receptors in target tissues. Thyroid system activity is regulated by a combination of factors including levels of T4 production, conversion of T4 to T3, hormone clearance rates, and levels of T3 receptors in target tissues (Dickhoff and Sullivan 1987). For example, it is possible that increases in thyroid activity in response to novel water exposure resulted in increased tissue levels of thyroid hormone with no observable change in plasma levels. Elevation in brain thyroid hormone concentration with no change in plasma level has been observed in yearling coho salmon (Specker, et al. 1992). Another possibility is that increased thyroid function in UW subyearling smolts may be reflected in higher T3 receptor levels rather than plasma T4 concentration. Recent reports have indicated that T3 receptor levels in the brain and olfactory epithelium increase during smolting, perhaps indicating an increased sensitivity to T3 in these tissues (Scholz et al. 1985; Yamauchi et al. 1993).

In conclusion, exposure to novel odorants or water sources did not elicit changes in plasma T4 levels in UW subyearling coho salmon at any developmental stage tested. Furthermore, UW subyearling coho salmon smolted but experienced no smolting associated plasma T4 surge. In light of the precise homing abilities of UW coho salmon reared under similar conditions, these findings suggest that elevated plasma T4 levels may not be critical for olfactory imprinting by coho salmon smolts.

FIGURE 5. Plasma T4 levels of subyearling coho salmon after exposure to either control water or 10^{-7} M PEA during the A) parr, B) smolt, or C) post-smolt stage in 1990. Vertical bars represent mean plasma T4 \pm standard error of the mean.

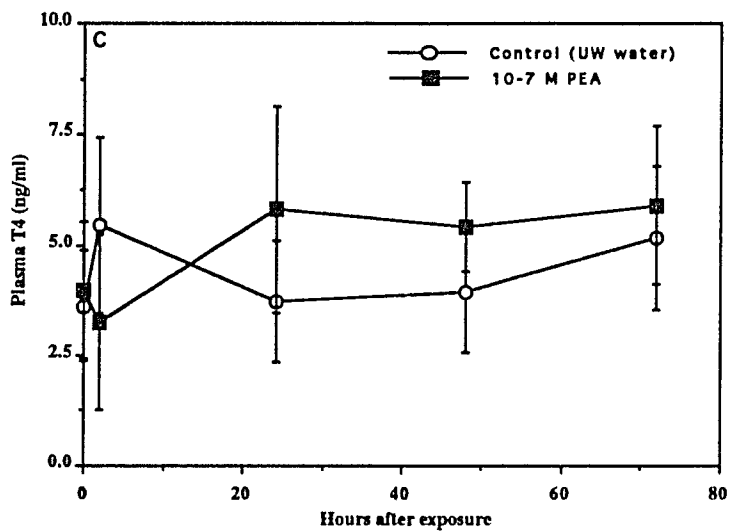
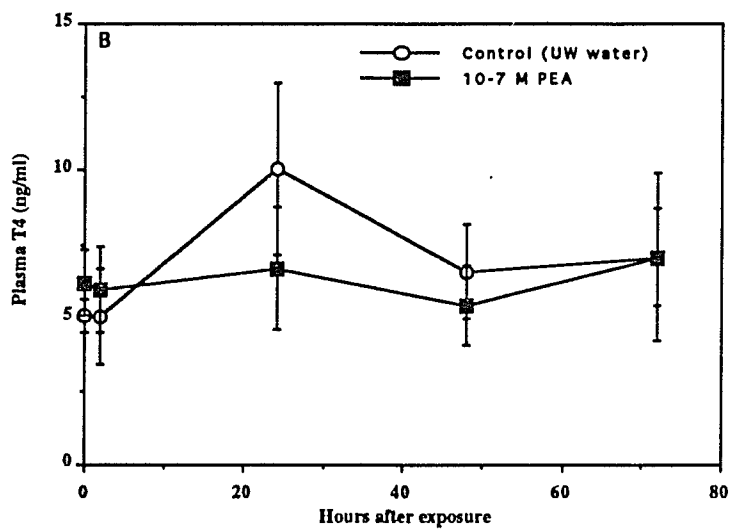
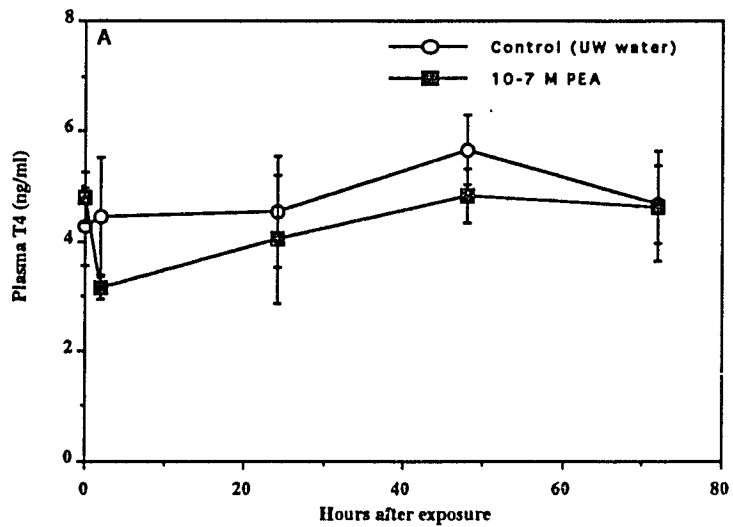


FIGURE 6. Plasma T4 levels of subyearling coho salmon A) parr and B) smolts after exposure to 10^{-7} M PEA or different levels of novel water in 1991. Vertical bars represent mean plasma T4 value \pm standard error of the mean.

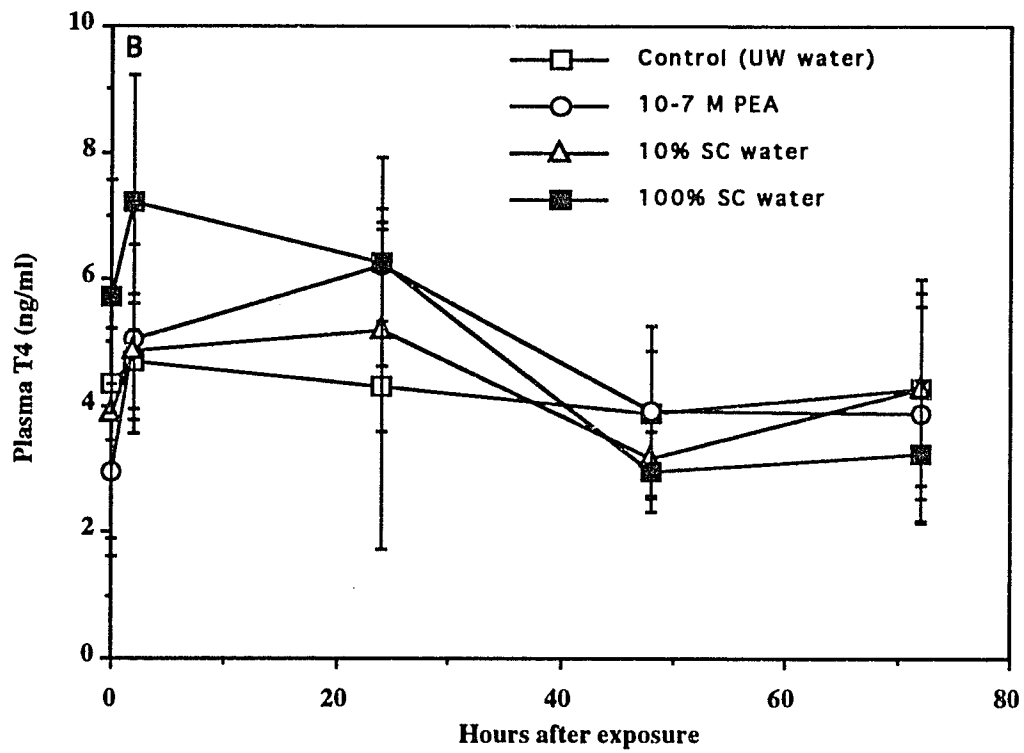
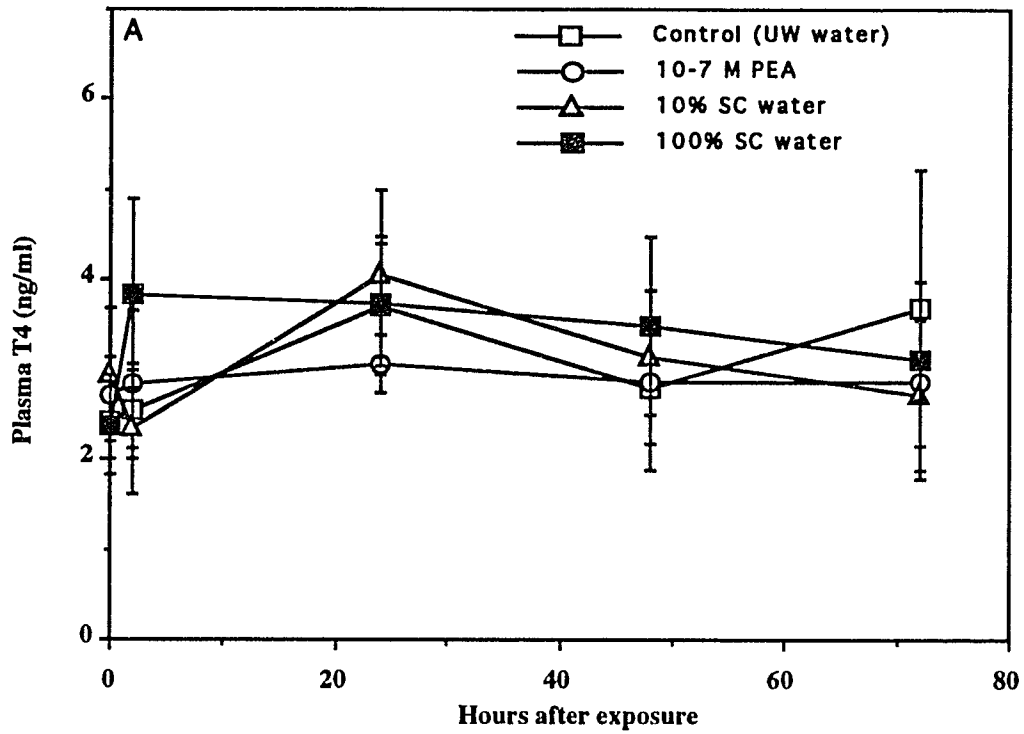


FIGURE 7. Plasma T4 levels of UW underyearling or yearling coho salmon during smoltification. Values for yearling coho salmon are from Sullivan (1986). Vertical bars represent mean value \pm standard error of the mean.

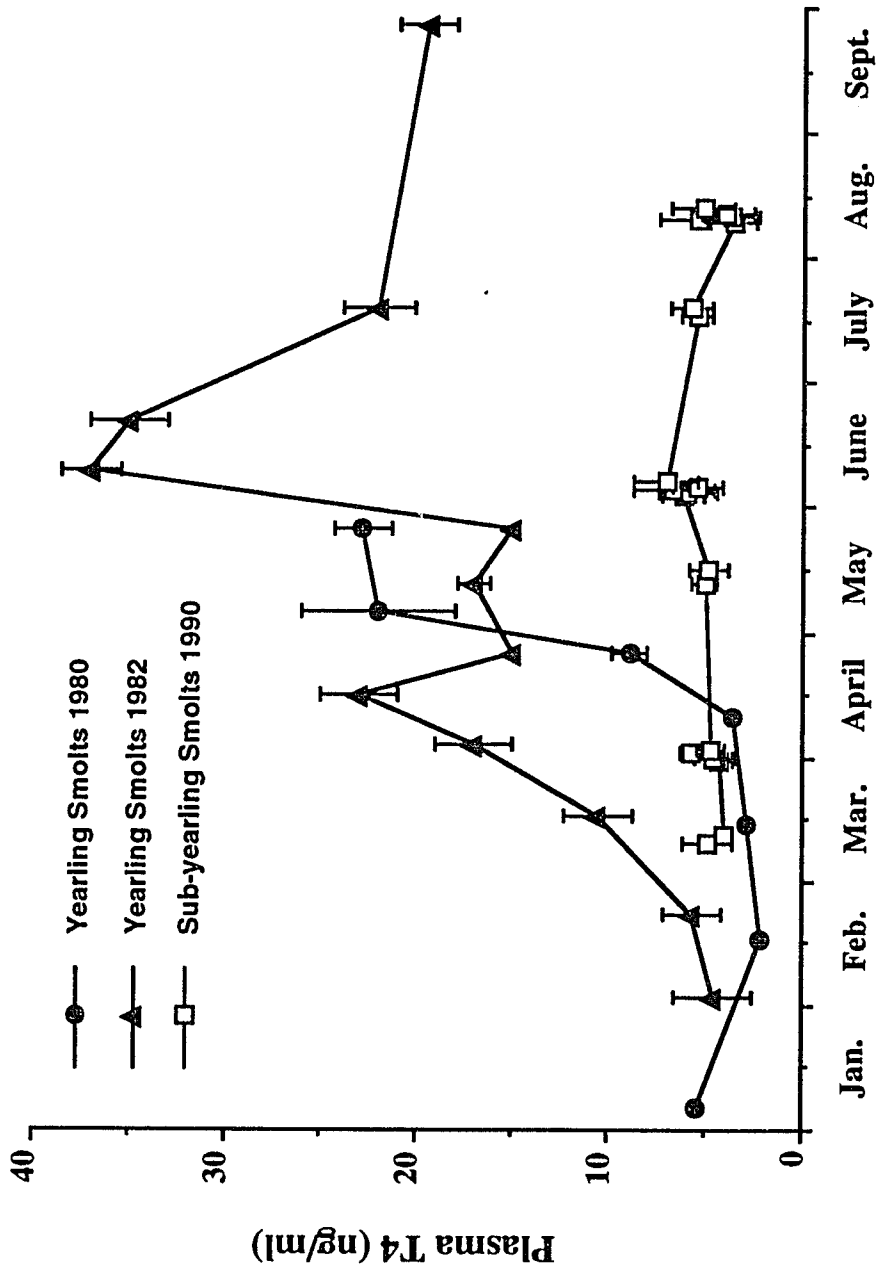


TABLE 3. Changes in plasma T4 concentration before and 48 hours after transfer of underyearling coho salmon from rearing tanks to experimental troughs containing identical UW water.

<u>Developmental stage/Date</u>	<u>Plasma T4 (ng/ml) before transfer</u>	<u>Plasma T4 (ng/ml) 48 h. after transfer</u>
Parr: March 10-12 1990	4.85 ± 1.31	4.03 ± 0.26
Smolt: May 13-15, 1990	5.03 ± 0.63	4.80 ± 1.00
Post-smolt: July 15-17, 1990	5.50 ± 0.80	5.70 ± 1.03
Parr: May 14-16, 1991	3.21 ± 0.49	3.46 ± 0.31
Smolt: May 14-16, 1991	5.11 ± 0.53	4.89 ± 0.33

Chapter III

Characterization and Odor Responses of Olfactory Adenylyl and Guanylate Cyclase from Imprinted Coho Salmon

Introduction

Pacific salmon (*Oncorhynchus spp.*) are known for their homing migrations from oceanic feeding grounds back to their river of origin to spawn. The final freshwater phase of these migrations is governed primarily by the olfactory discrimination of homestream water (Hasler and Scholz 1983). Prior to their seaward migration, juvenile salmon learn site-specific odors associated with their home stream and later use these odor memories for homing. This phenomenon, known as olfactory imprinting, was elegantly demonstrated by Hasler and his colleagues who found that juvenile coho salmon (*O. kisutch*) exposed to artificial odorants, such as morpholine and phenylethyl alcohol (PEA), could be lured into an unfamiliar stream scented with these odors during their spawning migration years later (Scholz et al. 1976; Cooper et al. 1976). Recently, we demonstrated that this long-term memory of imprinted odors in coho salmon may be due in part to sensitization of peripheral olfactory receptor neurons to specific odorants (Nevitt et al. 1994). The cellular mechanisms underlying imprinted odor recognition and receptor cell sensitization to imprinted odors are not known.

Odor detection in vertebrates is thought to be initiated by the interaction of odorants with specific G protein-coupled receptors on the cilia of olfactory receptor neurons (Buck and Axel 1991; Ngai et al. 1993). For many odors, receptor activation causes a rapid increase in the intracellular messenger cAMP, thought to directly activate cyclic nucleotide gated cation channels resulting in membrane depolarization and generation of action potentials (Pace et al. 1985; Sklar et al. 1986; Nakamura and Gold 1987; Breer et al. 1990). Increased cAMP production is G protein dependent and is apparently mediated via the odorant-sensitive type III adenylyl cyclase which is enriched in olfactory cilia (Bakalyar and Reed 1990). Other odorants which do not activate adenylyl cyclase can increase intracellular inositol triphosphate (IP3) levels, suggesting multiple transduction pathways in olfactory receptor neurons (Huque and Bruch 1986; Boekhoff et al. 1990). Odorant activation has also been linked to guanylate cyclase stimulation and increased levels of ciliar cGMP (Restrepo et al. 1993; Breer and Shepherd 1993).

The salmon olfactory system is extremely sensitive; thresholds for detection of odors such as amino acids, steroids and imprinted odorants range from nanomolar to femtomolar concentrations (Moore and Scott 1991; Andersen and Døving 1991; reviewed in Hara 1992). In salmon, odors are detected by microvillar and ciliar olfactory receptor cells, which together with structural and mucous secreting cells, make up the epithelial layer of the olfactory organ, the olfactory rosette. It is generally accepted that the initial events in odorant recognition and olfactory transduction occur in the apical dendritic membranes of these receptor cells (e.g. Rhein and Cagan 1980, 1983; Brand and Bruch 1992). While considerable effort has been directed at understanding the electrical properties of the salmon olfactory epithelium in response to odorants (reviewed in Hara 1992), little is known about the biochemical mechanism of odor recognition and signalling in salmon receptor neurons. Binding sites for amino acid odorants have been identified in dendritic membranes isolated from olfactory rosettes of several salmonid species (Rhein and Cagan 1983; Rehnburg and Shreck 1986; Lo et al. 1991). Lo et al. (1993) recently reported that phospholipase C activity in Atlantic salmon (*Salmo salar*) olfactory membranes is stimulated by amino acids in a G protein dependent manner. Nevitt and Moody (1992) characterized the electrical properties of ciliated olfactory receptor neurons in coho salmon and demonstrated that intracellular cGMP could modulate channel properties. Beyond these studies, little is known about transduction mechanisms involved in salmonid olfaction and particularly recognition of imprinted odorants.

As an initial step in understanding the molecular mechanisms underlying the transduction of imprinted odorants, we exposed juvenile coho salmon to the artificial odorant PEA and subsequently examined the regulation of cAMP and cGMP metabolism in PEA-exposed and PEA-naive coho salmon olfactory cilia. Specifically, we determined the sensitivity of cilia adenylyl and guanylate cyclase activity to various effectors including amino acids and PEA. We also determined whether olfactory adenylyl and guanylate cyclase were more sensitive to PEA in imprinted vs. PEA-naive fish.

Methods

Experimental animals

On December 1, 1987, we collected, pooled and fertilized 10,000 coho salmon eggs from adult salmon that returned to spawn at the University of Washington (UW) hatchery. Fish were reared at the UW until the following spring when they were separated into experimental and control groups. The experimental group was exposed to 10^{-7} M PEA for 10 days during smolt-transformation, a sensitive period for imprinting during which salmon undergo a physiological and behavioral transformation in preparation for life at sea. Experimental and control fish, that never experienced PEA, were given distinct marks, transferred to a freshwater rearing facility and reared communally until they matured in either fall 1989 or 1990. Successful imprinting was confirmed by behavioral assays and patch clamp experiments with olfactory receptor neurons isolated from PEA-exposed and PEA-naive fish (Nevitt et al. 1994; Chapter I)

Isolation of olfactory cilia

Olfactory cilia for adenylyl cyclase assays were isolated from mature PEA-naive and PEA-exposed coho salmon rosettes using the calcium shock procedure described by Chen et al. (1986) except where noted. All procedures were performed at 0-4° C and cilia were prepared fresh for each experiment. Briefly, rosette tissue was quickly excised and washed 3 times in ice-cold buffer A (100 mM NaCl, 2.0 mM EDTA, 30 mM Tris-HCl, pH 7.5, 1.0 mM PMSF, 0.5 µg/ml leupeptin, 1.0 µg/ml pepstatin A). This rosette suspension was rotated end-over-end for 20 min in the presence of 10 mM CaCl₂ to detach cilia, centrifuged for 5 min at 1500 x g and the resulting cilia-containing supernatant was centrifuged at 20,000 x g for 20 min. Precipitated cilia membranes were washed and resuspended in Buffer B (3.0 mM MgCl₂, 2.0 mM EDTA, 10.0 mM Tris-HCl, pH 7.5, 1.0 mM PMSF, 0.5 µg/ml leupeptin, 1.0 µg/ml pepstatin A). Membranes from ciliated and deciliated olfactory epithelium and other tissues were prepared by polytron homogenizing (60s, 0° C, setting 5) in Buffer A, centrifuging at 1000 x g to remove debris and then centrifuging the resulting supernatant at 20,000 x g. Pelleted membranes were resuspended in Buffer B and immediately assayed. Cilia and other membranes for guanylate cyclase assays were prepared as described above except all

buffers contained 10mM NaF and 10mM benzamide in place of PMSF. Protein concentrations were determined by the method of Peterson (1983).

Adenylyl cyclase assays

Adenylyl cyclase activity was determined by adding membranes (1.0-10.0 μg of protein) to an assay solution containing 1.0 mM α -[^{32}P]ATP (1000 cpm/pmol), [^3H]-cyclic AMP (20,000 cpm/ μmol), 5.0 mM MgCl_2 , 1.0 mM EDTA, 0.2 mM EGTA, 2.0 mM cAMP, 0.5 mM dithiothreitol, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 % bovine serum albumin, 20mM phosphocreatine, and 100 units/ml creatine phosphokinase and 20 mM Tris-HCl, pH 7.4 in a final volume of 250 μl . Except where noted, assays were performed at 22° C for 20 min. Assays were terminated by adding 750 μl 1.5% SDS, and the [^{32}P] cyclic AMP generated was measured as described by Salomon et al. (1974). All assays were performed in triplicate with freshly prepared cilia and membranes.

Guanylate cyclase assays

Guanylate cyclase activity was determined by the method of Nesbitt et al. (1976) with slight modifications. Reactions were initiated by adding membranes (1.0-10.0 μg of protein) to an assay solution containing 0.5 mM α -[^{32}P]GTP (2000 cpm/pmol), 2.4 mM cyclic GMP, 10.0 mM MgCl_2 or 2.5 mM MnCl_2 , 1.0 mM dithiothreitol, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 % bovine serum albumin, 20mM phosphocreatine, and 100 units/ml creatine phosphokinase in 50 mM Tris-HCl, pH 7.5, in a final volume of 100 μl . After 20 min at 22° C reactions were terminated by adding 0.5 ml of ice-cold stop solution (1.0 mM GTP, 5.0 mM [^3H] cyclic GMP (6000 cpm/ μmol)) and 50 μl 50% TCA. [^{32}P] cyclic GMP production was measured as described by Nesbitt et al. (1976). All assays were performed in triplicate with freshly prepared cilia and membranes.

Statistics

A two-tailed Student's t test was used to test for differences between mean values of adenylyl and guanylate cyclase activity in the presence or absence of odorants in cilia isolated from PEA-naive and exposed fish.

Results

Coho salmon olfactory cilia contained relatively high levels of adenylyl cyclase (150-300 pmol/mg/min) and guanylate cyclase (100-400 pmol/mg/min) activity. The basal rate of cAMP production in cilia was linear between 2-30 min and over a protein range of 0.2-15.0 μ g/assay. Cyclic GMP production was linear between 2-30 min and over a protein range of 0.5-20.0 μ g/assay. We anticipated that both enzymes would demonstrate optimal activity within a temperature range normally experienced by coho salmon (e.g. 10-15° C) as has been described for other ectothermic fish (Hochachka and Somero 1971). However, we observed maximal enzyme activity between 20-30° C, with significantly lower activity at 10° C for both enzymes. All subsequent assays were conducted at 22° C.

Basal and forskolin-stimulated adenylyl cyclase activity was enriched 2-2.5 and 4-6 fold, respectively, in olfactory cilia compared to membranes prepared from whole or deciliated rosettes (Figure 8A). Ciliar adenylyl cyclase activity was higher than in liver membranes and comparable to adenylyl cyclase activity in brain membranes, a particularly rich source of the enzyme in mammals (Brostrom et al. 1975). GTP had little effect on basal adenylyl cyclase activity in cilia but 100mM GppNHp, a non-hydrolyzable GTP analog, increased activity two-fold suggesting coupling to a stimulatory G protein (Figure 8A). Like the recombinant type III olfactory adenylyl cyclase (Bakalyar and Reed 1990), salmon olfactory adenylyl cyclase was particularly sensitive to forskolin (7.53 fold stimulation) compared to brain or liver cyclase (3.85 and 4.32 fold stimulation, respectively). While brain adenylyl cyclase was stimulated directly by Ca^{2+} /CaM, activation of the olfactory enzyme by Ca^{2+} /CaM required preactivation with GTP analogs (Figure 8A).

Guanylate cyclase activity was undetectable using Mg^{2+} -GTP as a substrate but low levels of guanylate cyclase activity were detectable in all tissues with Mn^{2+} -GTP. As in mammalian tissues (Steinlen et al. 1990), the addition of the non-ionic detergent, Lubrol PX, increased guanylate cyclase activity in all tissues and 15-fold in salmon olfactory cilia (Figure 8B). In the presence of Mn^{2+} /Lubrol PX, guanylate cyclase activity was enriched two-fold in cilia compared to membranes prepared from whole or deciliated rosettes (Fig 8B). Guanylate cyclase activity in brain membranes was approximately two-fold higher than in cilia.

If adenylyl cyclase activity in salmon olfactory cilia is important in olfactory signalling, we hypothesized that G protein-coupled enzyme activation would be specific for odorants. We tested several G protein-coupled hormones and neurotransmitters that stimulate adenylyl cyclase in other membranes and found that adenylyl cyclase in olfactory cilia was not affected by these compounds (Table 5). Within physiological ranges, amino acid odorants also had no effect on adenylyl cyclase activity but were slightly stimulatory at very high concentrations ($P < 0.03$ for $100 \mu\text{M}$ L-serine; $P < 0.05$ for $100 \mu\text{M}$ L-alanine) (Figure 9A). This stimulation was only observed in the presence of $20 \mu\text{M}$ added GTP. Because salmon have greater olfactory sensitivity for L- vs. D- amino acid isomers (Hara 1982), we tested whether L-serine and L-alanine were more effective agonists than their D isomers. For both amino acids, the L- and D- stereoisomers had similar effects on adenylyl cyclase activity except at the highest concentrations (Figure 9A). Guanylate cyclase activity was not affected by amino acids at any concentration tested (Figure 9B).

To test the effects of an imprinted odorant on olfactory adenylyl and guanylate cyclase activity, we examined enzyme activity in the presence of PEA in cilia isolated from the rosettes of mature salmon that were either PEA-naive or exposed to 10^{-7} M PEA as smolts. We also examined the effect of PEA on adenylyl and guanylate cyclase activity in brain membranes to determine the specificity of olfactory responses to PEA. As with L amino acids, PEA had no effect on cilia adenylyl cyclase activity at concentrations which elicit electrophysiological and behavioral responses but there was a slight increase in activity at high PEA concentrations ($P < 0.15$ for $100 \mu\text{M}$ PEA) (Figure 10A). Stimulation was only observed in the presence of $20 \mu\text{M}$ added GTP. Data from eight independent adenylyl cyclase experiments indicated that there was no difference in PEA sensitivity in PEA-exposed vs. PEA-naive cilia. Adenylyl and guanylate cyclase activity in brain membranes was not affected by PEA (Figure 10). In five of seven independent experiments, there was a slight but consistent increase in olfactory guanylate cyclase activity in both PEA-exposed and naive cilia at all PEA concentrations ($P < 0.05$) (e.g. Figure 10B). In two independent experiments using cilia isolated on Nov 3 and Nov. 6 1990, PEA elicited a dose-dependent 1.8-2.0 fold increase in guanylate cyclase activity only in cilia from salmon previously exposed to PEA ($P < 0.005$ for H_0 : No difference between PEA-naive and PEA-exposed cilia guanylate cyclase activity at 10^{-7} M PEA)(e.g. Figure 10C).

Discussion

Several lines of evidence suggest a possible role for adenylyl cyclase in coho salmon olfactory transduction. First, as in higher vertebrates, coho salmon olfactory cilia were enriched in adenylyl cyclase activity. Basal and forskolin-stimulated activity in olfactory cilia was approximately 0.2 and 2.0 nmol/mg/min, respectively, and comparable to levels in brain membranes. It should be noted, however, that basal levels of adenylyl cyclase activity were an order of magnitude lower than those described in isolated rat and frog olfactory cilia (Chen et al. 1985; Sklar et al. 1986). Second, although ciliary adenylyl cyclase is apparently sensitive to G stimulatory proteins, none of the common G protein-coupled hormone and neurotransmitters tested affected olfactory adenylyl cyclase suggesting a possible specificity of odorant receptors. Third, regulation of the adenylyl cyclase activity in salmon olfactory cilia was very similar to the mammalian type III olfactory adenylyl cyclase which has been implicated in olfactory transduction (Bakalyar and Reed 1990). Like the recombinant type III olfactory adenylyl cyclase, salmon olfactory adenylyl cyclase was particularly sensitive to forskolin (Bakalyar and Reed 1990) and activation of the olfactory enzyme by Ca^{2+} /CaM was synergistically enhanced by GTP analogs (Anholt and Rivers 1990; Choi et al. 1992). Fourth, while stimulation of adenylyl cyclase by amino acid and imprinted odorants was weak and required high odorant concentrations, the GTP-dependence of this phenomenon and greater stimulatory effectiveness of L- vs. D- amino acid isomers at high concentrations is consistent with adenylyl cyclase as a mediator of olfactory transduction. These results are consistent with electrophysiological findings with rainbow trout that L-serine and L-alanine elicited 2-4 x greater responses in the olfactory bulb than their D isomers (Hara 1975). Finally, while PEA elicited slight increases in adenylyl cyclase activity in cilia, it had no effect on adenylyl cyclase activity in non-olfactory tissue (e.g., brain membranes) at any concentration tested.

A physiological role for adenylyl cyclase in coho salmon olfaction remains uncertain, however, given the requirement for high odorant concentrations for only weak stimulation. Experimental determination of threshold concentrations required for amino acid and imprinted odorant signalling is variable depending on the assay method and tissue preparation used. Electrophysiological recordings from the olfactory bulb indicated that threshold concentrations for amino acid activation of the coho salmon olfactory system ranged from 10^{-6} to 10^{-7} M (Hara 1972). Olfactory-mediated avoidance of L-serine by coho salmon occurred at concentrations as low as 10^{-9} M but the threshold concentrations

required for behavioral responses depended on age and developmental stage of the test fish (Rehnberg et al. 1985). Amino acid binding studies with salmonid olfactory membranes gave mixed results but indicated K_d values ranging from 10^{-6} - 10^{-5} M for L serine and L alanine (rainbow trout: Rhein and Cagan 1980, 1983; Brown and Hara 1981; coho salmon: Rehnberg and Schreck 1986; Atlantic salmon: Lo et al. 1991). Experiments using fish from the same exposure groups as were used in the present study indicated that PEA-exposed coho salmon responded behaviorally to 10^{-7} M PEA and receptor neurons isolated from both PEA-naive and PEA-exposed fish responded to PEA at concentrations as low as 10^{-8} M (Nevitt et al. in press). Taken together, these results suggest that the concentrations of odorants required for only minimal amino acid and imprinted odorant stimulation of coho salmon adenylyl cyclase may be non-physiologically high.

Olfactory cilia from other fishes (e.g., catfish and Atlantic salmon) contained high levels of adenylyl cyclase activity but there was little indication of amino acid stimulation of adenylyl cyclase except at high odorant concentrations (Bruch and Teeter 1989, 1990; Lo et al. 1993). In contrast, GTP-dependent stimulation of ciliary IP3 levels by amino acids occurred at 10-100 fold lower concentrations (Bruch et al. 1987; Lo et al. 1993). It should be noted, however, that initial studies of rat and frog olfactory adenylyl cyclase indicated that *in vitro* stimulation of adenylyl cyclase required odorant concentrations exceeding physiological concentrations (10-100 μ M) using batch assay methods similar to those used in this study (Chen et al. 1985; Sklar et al. 1986). More recently, rapid kinetic measurements have confirmed that physiological concentrations of odorants elicit rapid (msec) and transient increases in cAMP in isolated rat cilia but over longer time-scales cAMP elevation is only apparent at high odor concentrations (Breer et al. 1990). Attempts to assay salmon adenylyl cyclase on a shorter time scale gave inconsistent results (Dittman, unpublished observation) and definitive resolution of adenylyl cyclase's role in amino acid and imprinted odor transduction may require more sophisticated stop-flow kinetic procedures.

The role of guanylate cyclase in vertebrate olfactory transduction is less clearly defined. Odorant activation of guanylate cyclase has been reported in rat and catfish cilia but cGMP increases were slower and longer lasting than cAMP and IP3 (Breer et al. 1992; Restrepo et al. 1993). Unlike catfish and Atlantic salmon cilia (Bruch and Teeter 1989; Lo et al. 1993), guanylate cyclase activity was slightly enriched in coho salmon olfactory cilia and displayed substrate specificity and detergent requirements that were similar to guanylate cyclase activity in rat and pig cilia (Steinlen et al. 1990). Amino acid

odorants had no effect on coho salmon cilia guanylate cyclase activity at any concentration tested. In contrast, 10^{-9} - 10^{-3} M PEA elicited slight increases in guanylate cyclase activity in cilia but not brain membranes isolated from PEA-naive fish.

What role might guanylate cyclase play in olfactory recognition of PEA by coho salmon? In other vertebrates, cyclic nucleotide-gated cation channels located in olfactory neurons are thought to mediate membrane depolarization in response to odorant-stimulated cAMP signalling (Nakamura and Gold 1987; Dhallan et al. 1990; Goulding et al. 1992). These channels are equally sensitive to cGMP and cAMP (Nakamura and Gold 1987; Goulding et al. 1992) and increased cGMP levels in response to PEA might lead to membrane depolarization and generation of action potentials. While cyclic nucleotide gated channels have been cloned from fish olfactory receptor cells, it is not known if similar channels are expressed in coho salmon receptor cells. Initial characterization of the electrical properties of coho salmon ciliated receptor cells by whole-cell patch-clamp recording indicated that relaxation of outward currents was slowed by cGMP application but the importance of this phenomenon in olfactory transduction is not known (Nevitt and Moody 1992).

Patch-clamp studies have indicated that olfactory receptor cells isolated from the PEA-imprinted salmon used in this study were more sensitive to PEA than cells from PEA-naive fish (Nevitt et al. in press). We hypothesized that this increased sensitivity might be reflected in the PEA sensitivity of adenylyl and guanylate cyclase activity in olfactory cilia isolated from PEA-naive and PEA-exposed salmon. Prior PEA exposure had no effect on adenylyl cyclase sensitivity to PEA and in five of seven guanylate cyclase experiments there was no difference in PEA sensitivity between PEA-exposed and naive cilia. In two experiments, however, PEA elicited a dose-dependent increase in guanylate cyclase activity in PEA-exposed cilia but not in PEA-naive cilia. Cilia for these experiments were isolated on Nov. 3 and 6, 1990, a period that coincided with maturation of the test fish. This effect was not apparent in subsequent experiments conducted two weeks later. If the data from all seven guanylate cyclase experiments are pooled, there was no significant difference in PEA sensitivity of guanylate cyclase from PEA-naive and imprinted fish. However, the results of the Nov. 3 and Nov. 6 experiments could reflect a narrow window of olfactory sensitivity associated with maturation. Olfactory sensitivity to specific odorants is affected by hormonal and maturational state. Coho salmon exposed to PEA and morpholine as juveniles only showed behavioral and electrophysiological responses to these odors upon maturation (Hasler and Scholz 1983). Similarly, the

olfactory system of precocious Atlantic salmon parr was only sensitive to a putative pheromone, testosterone, during a brief period just prior to spawning (Moore and Scott 1991). Further investigation will be required to determine if PEA sensitivity of guanylate cyclase is also controlled by maturational status.

FIGURE 8. Tissue distribution and regulation of adenylyl and guanylate cyclase activity in coho salmon. Membranes were prepared from mature 2 year old adults pooled from the PEA exposed and PEA-naive groups. Data are representative of three independent experiments. A) Membranes were assayed for adenylyl cyclase activity as described in the presence of 1) no added effectors; 2) 100 μ M GTP; 3) 100 μ M GppNHp; 4) 250 μ M CaCl₂ and 2.4 μ M CaM; 5) 100 μ M GppNHp, 250 μ M CaCl₂ and 2.4 μ M CaM; 6) 10 μ M Forskolin. B) Membranes were assayed for guanylate cyclase activity as described in the presence of 1) 2.5 mM MnCl₂ 2) 2.5 mM MnCl₂ and 0.5% Lubrol PX. No guanylate cyclase activity was detected in any tissue in the presence of 10 mM MgCl₂.

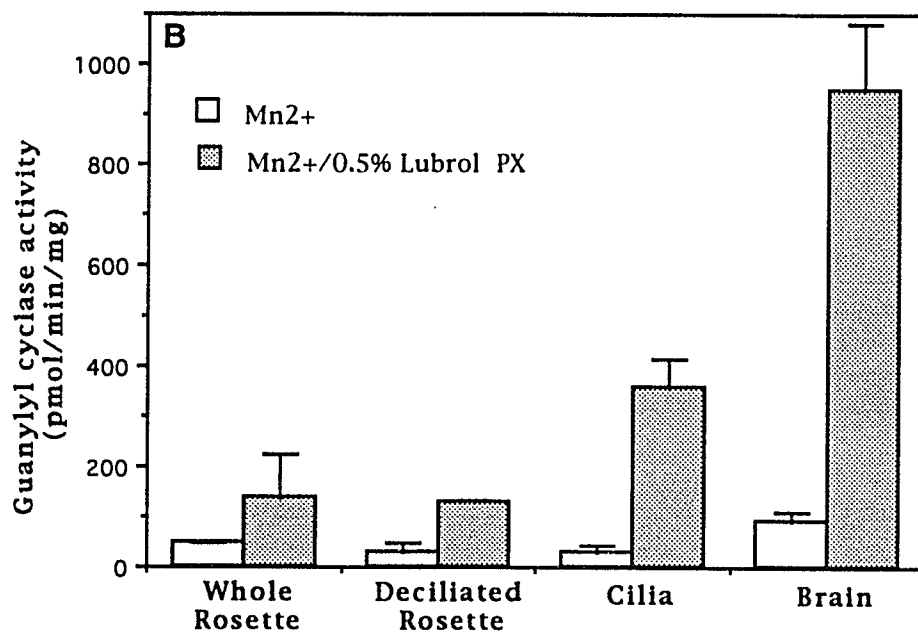
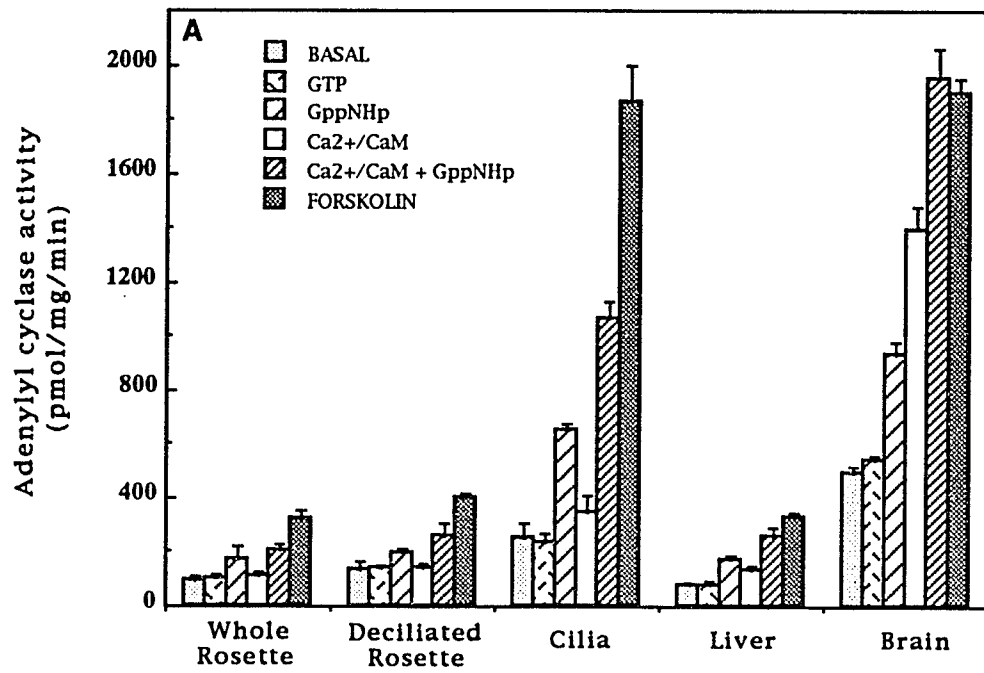


FIGURE 9. Fold stimulation of cilia adenylyl and guanylate cyclase activity in response to increasing amino acid odorant concentrations. Cilia were prepared from mature 2 or 3 year old adults pooled from the PEA exposed and PEA-naive groups. A) Membranes were assayed for adenylyl cyclase activity as described in the presence of 20 μ M GTP and amino acids as indicated. Data are representative of five independent experiments. Basal activity was 198.3 ± 4.9 pmol cAMP formed/mg/min. B) Membranes were assayed for guanylate cyclase activity as described in the presence of 2.5 mM MnCl₂, 0.5% Lubrol PX and amino acids as indicated. Data are representative of three independent experiments. Basal activity was 483.2 ± 75.7 pmol/cGMP formed/mg/min.

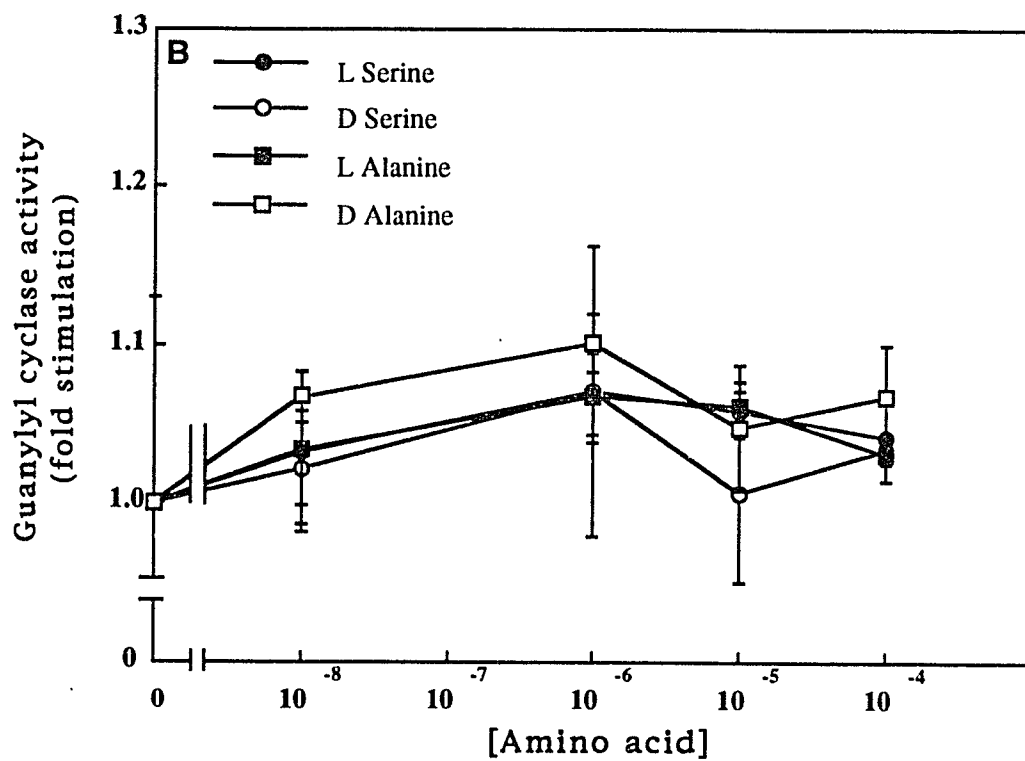
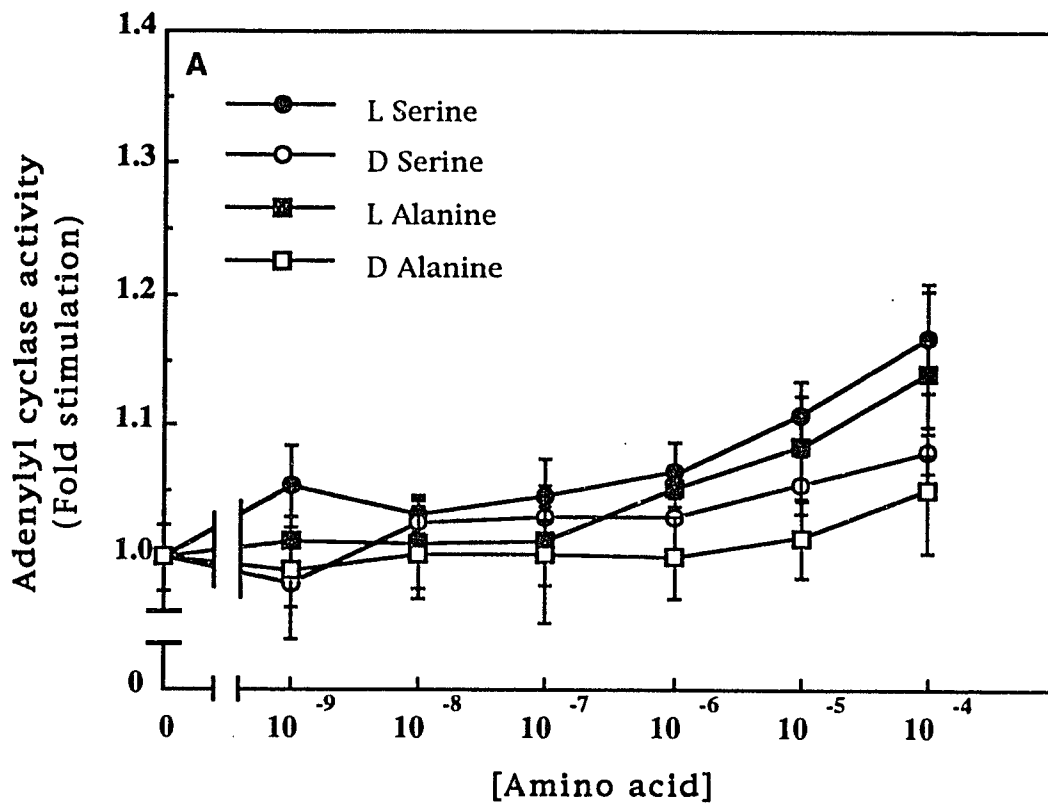


FIGURE 10. Fold stimulation of adenylyl and guanylate cyclase activity in response to increasing PEA concentrations in cilia isolated from PEA-exposed and naive salmon. Cilia were prepared from 10 two year old or 5 three year old mature PEA-exposed or PEA-naive salmon. Brain membranes were prepared from PEA-exposed fish. A) Membranes were assayed for adenylyl cyclase activity as described in the presence of 20 μ M GTP and PEA as indicated. Data are representative of eight independent experiments. Basal activities in exposed cilia, naive cilia and brain membranes were 304.3 ± 14.6 , 293.0 ± 8.5 , and 673.5 ± 33.9 pmol cAMP formed/mg/min, respectively. B) Membranes were assayed for guanylate cyclase activity as described in the presence of 2.5 mM MnCl₂, 0.5% Lubrol PX and PEA as indicated. Data are representative of five of seven independent experiments. Basal activities in exposed cilia, naive cilia and brain membranes were 583.2 ± 75.7 , 542.3 ± 41.8 , and 947.5 ± 131.9 pmol/cGMP formed/mg/min respectively. C) Membranes were assayed for guanylate cyclase activity as described in the presence of 2.5 mM MnCl₂, 0.5% Lubrol PX and PEA as indicated. Data are representative of two independent experiments using cilia isolated on Nov. 3, and Nov. 6, 1990. Basal activities in exposed cilia, naive cilia and brain membranes were 521.6 ± 37.0 , 505.9 ± 39.8 , and 892.4 ± 79.4 pmol/cGMP formed/mg/min respectively.

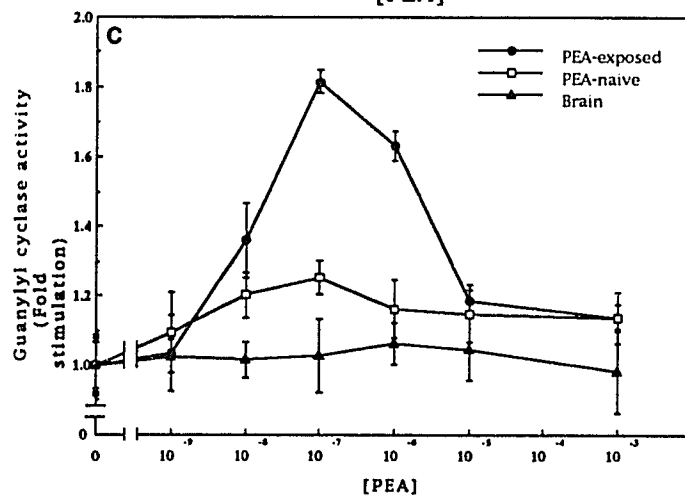
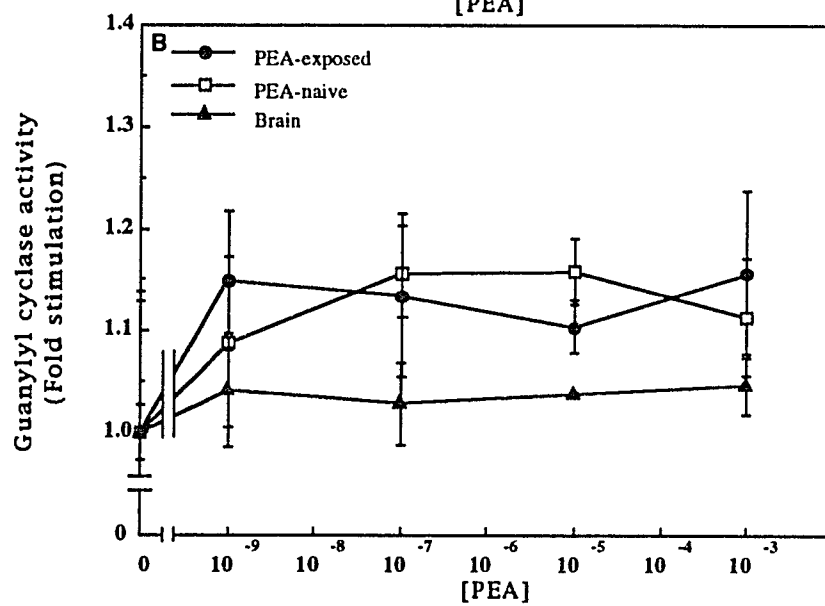
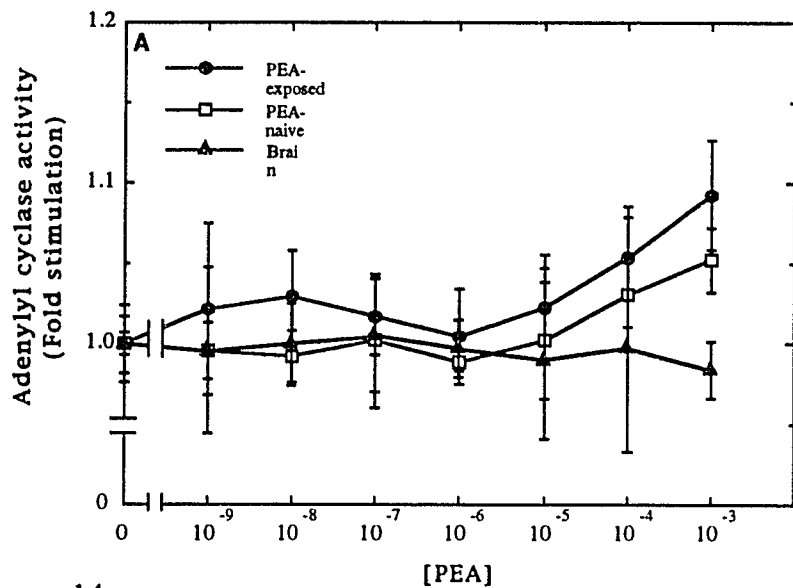


TABLE 4. Responses of cilia adenylyl cyclase to common hormone and neurotransmitter effectors. Membranes were assayed for adenylyl cyclase activity as described in the presence of 20 μ M GTP and 100 μ M effector as indicated. Controls were assayed in the presence of 20 μ M GTP alone. Data are representative of two independent experiments.

<u>TREATMENT</u>	<u>ADENYLYL CYCLASE ACTIVITY</u> <u>(pmol/mg/min)</u>
CONTROL	222.6 \pm 34.2
ISOPROTERENOL	230.0 \pm 51.7
NOREPINEPHERINE	212.5 \pm 82.6
DOPAMINE	241.3 \pm 63.5
EPINEPHERINE	267.2 \pm 26.2
HISTAMINE	197.3 \pm 58.7

Chapter IV

Olfactory Recognition and Behavioral Responses of Precociously Mature Salmon to a Putative Pheromone

Introduction

Olfactory cues emanating from conspecifics play an important role in social interactions between fishes including species, sex, kin, and population recognition, schooling, and predator avoidance (Liley 1982; Olsen 1992). For many fishes odors are also critical during reproduction, with pheromones emanating from both males and females eliciting behavioral and physiological changes which facilitate spawning. For a few species the chemical nature of these pheromones and their role in regulating mate attraction and spawning synchrony are being elucidated (e.g., Sorensen 1992a, 1992b; Stacey et al. 1993) However, the role of pheromones in salmonid reproduction and spawning behavior is still poorly understood (Olsen and Liley 1993; Rouger and Liley 1993).

During the final stages of their homing migration, salmon use olfactory cues to return to their river of origin to spawn. Hasler and Wisby (1951) proposed that juvenile salmon learn a unique bouquet of odors comprised of chemicals from rocks, soils, and plants in their homestream as homing cues. In contrast, Nordeng (1971, 1977) proposed that adult salmon are guided by population-specific odors emanating from juveniles in freshwater or migrating to sea. Adult salmon are attracted to water containing juvenile conspecifics (Selset and Døving 1980; Quinn et al. 1983) and salmon can distinguish between populations based on chemical cues (Olsen 1986; Groot et al. 1986; Quinn and Tolson 1986). However, field experiments have failed to demonstrate that homing salmon require the presence of members of their population at the natal site. Salmon will home to artificial odorants (Hasler and Scholz 1983) and are not decoyed to a non-natal site by the presence of fish from their population (Black and Dempson 1986; Brannon and Quinn 1990). Upon reaching their spawning grounds, however, odors cues from conspecifics may be involved in finding and courting potential mates.

Several lines of evidence indicate that salmon on the spawning grounds release and respond to odorants from the opposite sex. Mature male salmon are attracted to water scented by females in spawning condition or their ovarian fluid (Newcombe and Hartman 1973; Emanuel and Dodson 1979; Honda 1980, 1982; Olsen and Liley 1993). This

attraction was specific to ovulating females and experiments using anosmic males indicated that olfaction mediated these responses (Honda 1980, 1982; Olsen and Liley 1993). Odorants emanating from ovulating females were also able to "prime" males for spawning by stimulating production of gonadal steroids and increasing milt production (Liley et al. 1991; Olsen and Liley 1993). However, the chemical nature of the odorant(s) released by spawning females is unknown.

To date, sex pheromones identified in fishes have been primarily steroid hormones involved in gonadal maturation or their metabolites (Liley 1982; Sorensen 1992a). Therefore, we hypothesized that reproductive steroid hormones would also be the most likely salmonid sex pheromones. Furthermore, since pheromonal compounds that attract males are apparently only present in significant levels in ovulating females, we hypothesized that the most likely pheromone candidates would be hormones which are expressed at high levels at or near the time of ovulation and spawning readiness. While the hormonal regulation of gonadal development is complex, involving many steroidal and non-steroidal compounds, plasma concentrations of two reproductive hormones, gonadotropin (GtH) and $17\alpha,20\beta$ -dihydroxy-4-pregnene-3-one ($17\alpha,20\beta$ -P), rise dramatically in females at the time of spawning (Scott et al. 1983; Ueda et al. 1984; Dye et al. 1986; Liley et al. 1986a). The steroid $17\alpha,20\beta$ -P has been implicated as the major hormone responsible for final oocyte maturation (Goetz 1987) and acts as a potent pheromone mediating spawning synchrony in goldfish, *Carassius auratus* (Dulka et al. 1987).

In this study we tested whether $17\alpha,20\beta$ -P acts as a pheromone for male chinook salmon (*Oncorhynchus tshawytscha*) by measuring the behavioral responses of mature males to water scented with $17\alpha,20\beta$ -P as they returned to the University of Washington hatchery (UW) to spawn. To determine if these responses were important in reproductive behavior and not a general phenomenon for all life stages, we compared the responses of mature and immature male salmon to this odorant. To examine the specificity of responses to $17\alpha,20\beta$ -P, we also tested a second steroid hormone, testosterone, which is a potent attractant for mature male Atlantic salmon (*Salmo salar*) parr (Moore and Scott 1991; Moore 1991).

Methods

Experimental animals

Precociously mature male chinook salmon (i.e., mature in the first year only 5-6 months after release) were collected from the UW hatchery pond, from mid-October to early November in 1990 (150 fish; mean weight \pm s.e.: 61.90 ± 2.85 g) and 1991 (300 fish; 165.40 ± 3.83 g). These males were used because their small size facilitated behavioral testing. Experiments with large adult UW chinook males were not performed because of the limited number available and the cost of the odorants that would be required for testing large fish. Chinook salmon generally mature at three or four years old but in recent years up to 50% of the UW chinook salmon have matured precociously. Immature UW chinook salmon of the same age as the mature fish were reared at the UW hatchery until smolting, when they were transferred to another freshwater facility in the Lake Washington watershed. On November 9, 1990, 200 of these immature salmon (mean weight 124.69 ± 3.39 g) were transferred to UW for testing.

All test fish were maintained in water pumped from the Lake Washington Ship Canal in an outdoor concrete raceway for up to one week prior to testing. Mature fish were not fed prior to testing but immature fish were fed to satiation once a day up to 12 h before testing. Forty eight hours before testing, 30-50 fish were transferred to circular flow-through holding tanks in the laboratory for acclimation in Ship Canal water on a natural photoperiod. In 1990, 98 immature and 89 zero-age maturing male chinook salmon were tested for their responses to $17\alpha,20\beta$ -P and control water. In 1991, 272 mature males were tested for responses to $17\alpha,20\beta$ -P, testosterone, and control water.

Experimental apparatus

Fish were tested for water preference in two fiberglass two-choice tanks (140 x 52 cm) similar to those described by Quinn and Busack (1985). To ensure that the current characteristics were equivalent in each arm, water was spilled through identical V-notched walls from a small reservoir at the head of each arm. Tanks were filled to a depth of 8.0 cm and received a constant flow of 5.0 L/min/arm. All preference tests were conducting using untreated Ship Canal water. Stock solutions of test odorants were introduced into a test arm via a remotely operated peristaltic pump as the water spilled through the v-notch to facilitate maximum mixing. $17\alpha,20\beta$ -P (Sigma Chemical Co.) and testosterone (UW Pharmacy Services) stock solutions were made the day of testing by dissolving 1.0 mg of $17\alpha,20\beta$ -

P or 0.87 mg testosterone in 1.5 ml of methanol and subsequently diluting into 300 ml of distilled water to a final concentration of 10^{-5} M steroid. "Control water" solution consisted of 1.5 ml of methanol per 300 ml of distilled water. Stock solutions were metered into one arm of the maze at 5.0 ml/min to give a final concentration in the odor arm of 10^{-8} M steroid.

Testing procedure

To initiate a trial, an individual fish was placed in the screened downstream section of each tank and allowed to acclimate for 3 min. All observations were made through a slit in a black plastic curtain suspended behind the downstream end of the tanks. One minute before the end of the acclimation period, odorant was introduced to one arm of the tank. Dye tests indicated that odorant reached the downstream section of the tank in less than 1 min and considerable mixing occurred between the two sides of the downstream portion of the tank when fish were active. After acclimation, the screens were lifted and fish were allowed to swim freely within the tank. The first arm entered and final position (right arm, left arm or downstream area (no choice)) was recorded for each trial. The time spent by each fish in the left or right arm was also recorded during this 4 min test period. After testing, the mature fish were removed, measured (fork length), weighed and tested for spermiation by applying gentle pressure to the abdomen. Hatchery-raised immature fish were weighed, measured and dissected to determine sex and gonadal development. After each trial, tanks were drained, scrubbed with ethanol, rinsed thoroughly with Ship Canal water and the odorant supply tube was shifted to the other arm. Only one odorant was tested per day and the test odorant was varied daily to control for changes in behavioral responsiveness over the 3-4 week testing period. After each day of testing tanks were drained, scrubbed with ethanol and rinsed overnight with water.

Data Analysis

Preference for odorant-scented water was determined by four non-independent measures: first arm entered by the fish; position of the fish at end of trial; arm in which the fish spent the majority of time and proportion of time spent in each arm. Fish which entered neither arm during the course of the 4 min trial (no choice) were not included in the preference analysis. Differences in the proportion of fish making no choice in the presence of odorant were tested by χ^2 contingency tables (Zar 1984). To ensure that there was no bias associated with the experimental tanks, left and right side choices and time preferences

in each tank were pooled and compared to a 1:1 ratio expected by random choice. The proportion of time spent in each arm of the Y maze was analyzed by normalizing the data using an arcsine square root transformation and then using a two-tailed paired t-test to test for departure from the null hypothesis of no preference (Zar 1984). The first and last choice of all fish and arm in which they spent the majority of time in response to odorant were compared with the 50:50 distribution expected by chance using the χ^2 test. Where indicated, pairwise comparisons between experimental groups were tested using the comparisons of proportions test described by Zar (1984). In all cases, departures from no preference were tested at a significance level of $P=0.05$.

Results

Of the 50 mature zero-age males tested with Ship Canal water vs. Ship Canal water plus control water solution, only 2 made no choice and the remaining 48 fish displayed no preference for either water source by any of the measurements (Table 5). These results indicated that the methanol-containing distilled water carrier solution did not influence the subsequent tests with steroid odorants. For all test groups and all odor treatments, final position did not differ from a 50:50 no preference response. Responses of mature males to $17\alpha,20\beta$ -P were tested in both 1990 and 1991 but there were no differences between years (for all measures of preference and proportion of fish making no choice, $p > 0.20$) and data were pooled for further analysis. Of the 161 fish tested, 88% were spermiating. Mature males avoided the the arm of the maze containing 10^{-8} M $17\alpha,20\beta$ -P, based on the first arm chosen, arm in which fish spent the majority of time, and proportion of time spent in each arm (Table 5, Fig. 11). These measures were also significantly different than the responses of mature males to control water and testosterone and the response of immature fish to $17\alpha,20\beta$ -P.

To test whether avoidance of $17\alpha,20\beta$ -P was restricted to mature males, we also tested hatchery-reared UW chinook males of the same age but with little gonadal development. Of the 183 immature fish tested, 98 were males which had a slight but non-significant tendency to prefer the $17\alpha,20\beta$ -P arm (Table 5, Fig. 11). No difference was apparent between immature males and females by any of the four measures ($p > 0.10$ for all measures). In 1991, we also tested the behavioral responses of 149 mature males to 10^{-8}

M testosterone. They demonstrated no preference for or avoidance of testosterone by any measure. Of the testosterone test fish, 97% were spermiating.

In the presence of either steroid, mature salmon were less likely to move upstream into either arm than either immature fish in the presence of steroid or mature fish in the presence of control water as indicated by the number making no choice during a trial. The proportion of mature fish making no choice in the presence of $17\alpha,20\beta\text{-P}$ (38%) was higher than either immature fish in the presence of $17\alpha,20\beta\text{-P}$ (18%, $\chi^2=10.05$, $p<0.005$) or mature fish in the presence of control water (2%, $\chi^2=17.95$, $p<0.001$). Similarly, the proportion of mature fish making no choice in the presence of testosterone (34%) was higher than mature fish in the presence of control water (2%, $\chi^2=15.44$, $p<0.001$). In addition to this decreased positive rheotaxis, mature fish in the presence of steroid were generally less active than either immature fish in the presence of steroid or mature fish in the presence of control water.

Discussion

We hypothesized that the reproductive steroid hormone, $17\alpha,20\beta\text{-P}$, might act as a pheromonal attractant for mature male salmon but precocious male chinook salmon avoided water scented with this putative pheromone. These results were unexpected since mature males are generally attracted to ovarian fluid of ovulating females and these fluids contain high levels of $17\alpha,20\beta\text{-P}$. For example, $17\alpha,20\beta\text{-P}$ levels in ovulating coho salmon females may reach concentrations as high as 10^{-7} M in ovarian fluid (Wright and Hunt 1982). There are several possible explanations for the apparent avoidance of this steroid. First, avoidance of $17\alpha,20\beta\text{-P}$ may be a consequence of the concentration of the odorant tested and at this concentration both precocious and adult males might avoid $17\alpha,20\beta\text{-P}$. Second, ovarian fluid may contain attractive compounds which overcome the repulsive properties of $17\alpha,20\beta\text{-P}$. Third, $17\alpha,20\beta\text{-P}$ may only be attractive as part of a mixture of pheromones released by females. Finally, since experimental evidence for attraction to ovarian fluid has not been established in chinook salmon, the responses of chinook salmon males may differ from other salmonid species tested. This seems unlikely; mature male UW chinook salmon were attracted to water scented by females (Pete 1977). These results are apparently not due to a generalized avoidance by chinook salmon of $17\alpha,20\beta\text{-P}$ in this testing paradigm. Avoidance of $17\alpha,20\beta\text{-P}$ was specific for mature males and was not

observed for immature males of the same age. This suggests that $17\alpha,20\beta$ -P is a behaviorally significant odorant for reproductively active male chinook salmon.

The avoidance of $17\alpha,20\beta$ -P may reflect differences in the behavioral responses to pheromones by precociously maturing and adult males. Previous studies describing male attraction to females have only tested large adult salmon and not precociously maturing males (Newcombe and Hartman 1973; Emanuel and Dodson 1979; Honda 1980, 1982; Olsen and Liley 1993). On spawning grounds, precocious and larger chinook males employ dramatically different tactics for gaining access to females (Gebhards 1960). Adult males actively court nest-building females and aggressively defend females from other males. Precocious males generally employ sneaking tactics, remaining in the vicinity of females, not engaging in male-male aggression but attempting to sneak fertilizations (Gebhards 1960; Gross 1985; Maekawa and Onozato 1986; Hutchings and Meyer 1988). Males adopting this sneaking tactic might respond differently to pheromonal communication from females. While adult males actively seek ovulating females, precocious males may actually avoid high concentration of $17\alpha,20\beta$ -P because close proximity to females might invite attacks from both males and females (Hanson and Smith 1967; Gross 1985). However, the advantage of such an avoidance response in the absence of competing males is not clear, since precocious males of other salmonid species will actively court large females in the absence of competitors (Foote and Larkin 1988).

Another explanation is that $17\alpha,20\beta$ -P avoidance reflects pheromonal communication between males. As in females, levels of $17\alpha,20\beta$ -P increase dramatically in male salmon at the onset of spawning (Ueda et al. 1984; Dye et al. 1986; Liley et al. 1986b). Mature adult males that are subsequently paired with nest-building females exhibit even further elevations in $17\alpha,20\beta$ -P levels (Liley et al. 1986b; Liley et al. 1991; Olsen and Liley 1993; Rouger and Liley 1993). These socially-induced increases in $17\alpha,20\beta$ -P may increase milt production and synchronize spawning (Rouger and Liley 1993) but may also be important for pheromonal signalling between males. Just as physical attributes such as body length and depth act as fitness indicators in establishing dominance among males (Quinn and Foote, in press), olfactory cues such as gonadal hormones may also be involved in status signalling. This might explain the avoidance of high $17\alpha,20\beta$ -P concentrations by precocious males since their levels of $17\alpha,20\beta$ -P are lower than those of adult males during spawning (Stuart-Kregor et al. 1981; Ueda et al. 1983). Such a system might be particularly important at night, when visual cues are lacking but spawning activity continues (e.g. Hartman 1969).

The spawning tactics of precocious males may also explain the decreased activity and positive rheotaxis (as indicated by percentage of fish making no choice) of test fish in the presence of $17\alpha,20\beta$ -P vs. control water. On the spawning grounds zero-age males avoid attacks by limiting activity and remaining inconspicuous to females and larger males (Gebhards 1960). Since both adult males and females may release $17\alpha,20\beta$ -P into the surrounding water while courting, limiting activity in the presence of $17\alpha,20\beta$ -P may minimize attacks. However, this does not explain the decreased activity levels we also observed in the presence of testosterone.

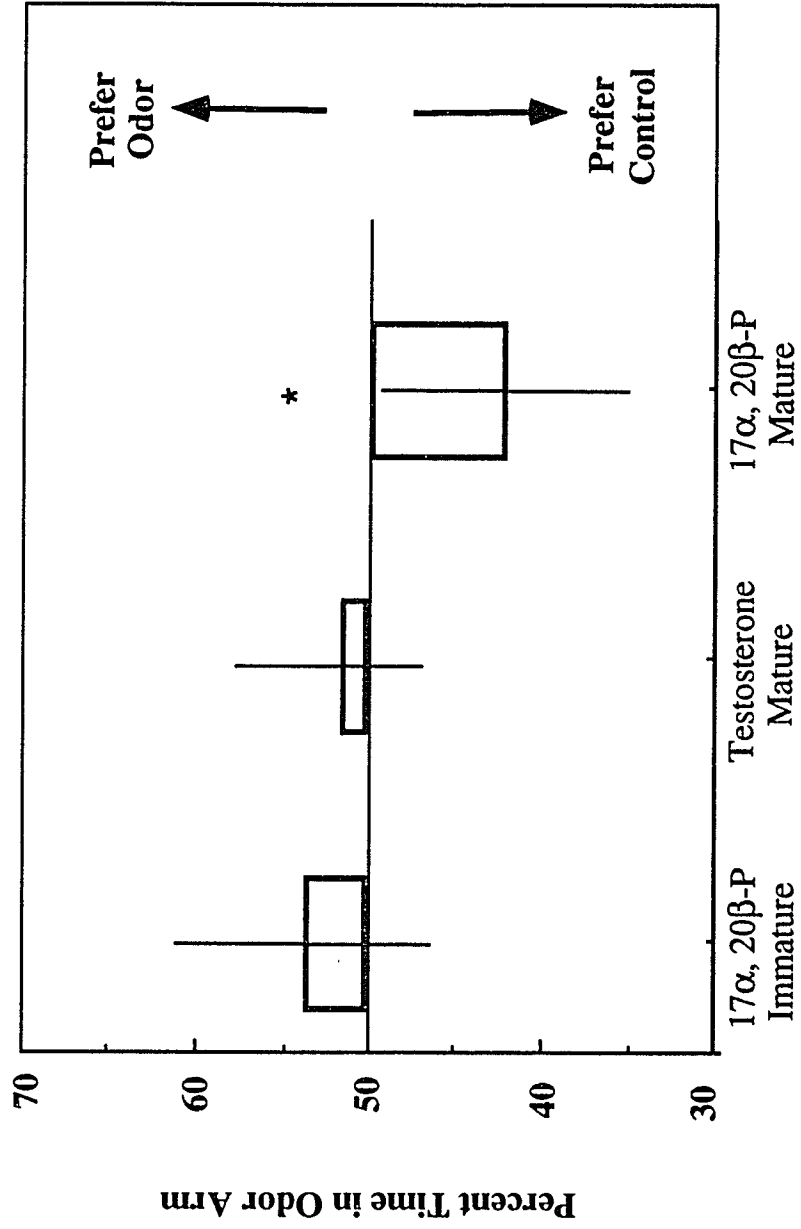
Precocious male salmon demonstrated no behavioral preference for or avoidance of testosterone. This indicated that the observed responses to $17\alpha,20\beta$ -P were not due to a general avoidance of steroids. Precocious males were able to recognize testosterone because the percentage of fish making no choice increased in the presence of this steroid. In contrast to our results, mature Atlantic salmon parr exhibited a strongly positive rheotactic response to water scented with testosterone (Moore 1991). Furthermore, electrophysiological recordings of the olfactory epithelium indicated that testosterone is an extremely potent odorant for mature parr (Moore and Scott 1991). Surprisingly, physiological recognition of testosterone was limited to a narrow time window just prior to spawning. This limited window of sensitivity might explain why we saw no behavioral responses to this odorant or alternatively this may reflect differences between the species.

Since we initiated this study several researchers have also suggested that the sulphated conjugate of $17\alpha,20\beta$ -P, $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one 20-sulphate, which is present in high levels in the plasma and urine of several teleosts (Scott and Canario 1992), may act as a sex pheromone (Stacey et al. 1993). This compound is a potent odorant for goldfish (Sorensen et al. 1991) and male Atlantic salmon parr (Moore and Scott 1992). While we did not test the responsiveness of precociously mature chinook males to $17\alpha,20\beta$ -P-sulphate, Scott et al. (1994) found that mature male rainbow trout neither avoided nor were attracted to this compound.

In summary, precociously maturing zero-age chinook salmon avoided water scented with the reproductive steroid hormone $17\alpha,20\beta$ -P. This avoidance response was not apparent in immature male or female chinook salmon of the same age or mature males tested in the presence of a second steroid hormone, testosterone. Taken together, these results suggest that $17\alpha,20\beta$ -P is a behaviorally relevant odorant for precociously maturing male salmon. We hypothesize that large mature male salmon would be attracted to

$17\alpha,20\beta$ -P and the avoidance responses we observed were associated with the sneaker spawning tactics employed by precocious fish.

Figure 1. Behavioral responses of immature and precociously mature chinook salmon to water scented with 17α , 20β -P and testosterone. Data represents mean percentage time spent in the arm scented with odorant vs time spent in the control arm. Significant departures ($p=0.05$) from the 50 % level of no preference are indicated by an asterisk. Vertical lines represent the 95% confidence intervals back calculated from arcsine transformations used for statistical analysis.



Experimental Group

Table 5. Responses of mature and immature chinook salmon to water scented with 17α , 20β -P, testosterone, or control water. Data represent responses of individual fish (odor arm, control arm (Ctl), or no choice (NC)) as measured by first arm entered, position at end of 4-min trial, and arm in which fish spent the majority of time (Time Preference). Significant departures from a 50:50 no preference response are indicated by asterisks.

	Odorant Tested	First Arm Entered			Final Position			Time Preference		
		Odor	Ctl.	NC	Odor	Ctl.	NC	Odor	Ctl.	NC
Mature	Control water	24	24	2	23	25	2	26	22	2
Mature	17α , 20β -P	38	62*	61	39	41	81	40	60**	61
Immature	17α , 20β -P	44	36	18	40	40	18	34	46	18
Mature	Testosterone	57	42	50	32	36	81	49	50	50

* $\chi^2= 5.76$, $P<0.05$:

** $\chi^2= 4.00$, $P<0.05$:

Chapter V

Regulation of the Ca^{2+} /Calmodulin Sensitive Type III Olfactory and Type I Adenylyl Cyclase by G Protein Coupled Receptors

Introduction

The family of muscarinic acetylcholine receptors (m1-m5) couple via guanine nucleotide binding proteins (G proteins) to a variety of signal transduction systems within cells including adenylyl cyclases, phospholipase C, and ion channels (Nathanson 1987; Jones et al. 1992). Tissue-specific responses to these receptors are governed by the complex interactions between distinct receptor subtypes, G protein subunits, and effectors expressed in different cell types. Within a given cell, individual muscarinic receptor subtypes can activate multiple G protein/effector pathways adding further complexity to this system. Consequently, a specific muscarinic receptor subtype can initiate divergent intracellular processes when expressed in different cell or tissue types. For example, in most neuronal tissues, m4 muscarinic receptors couple to inhibition of adenylyl cyclase activity, but in the olfactory bulb, m4 activation stimulates adenylyl cyclase activity (Olianas and Onali 1990, 1991).

What determines the tissue specificity of these responses? While considerable effort has been directed at this question by examining the role of receptor/G protein specificity (Ross 1989; Hille 1992), until recently, the role of distinct adenylyl cyclase isozymes in determining specific muscarinic responses has been less thoroughly investigated (Tang and Gilman 1991; Federman et al. 1992; Duzic and Lanier 1992). With the recent cloning and characterization of six distinct adenylyl cyclase isozymes, however, the breadth of regulatory diversity represented in the adenylyl cyclase family is now being recognized (reviewed in Choi et al. 1993a). Although it was originally believed that only the alpha subunits of G_s or G_i could directly regulate adenylyl cyclases, there is now evidence that the beta/gamma complex can also modulate adenylyl cyclase activity (Tang and Gilman 1991; Federman et al. 1992; Taussig et al. 1993). All six adenylyl cyclases cloned to date are sensitive to G_s -alpha stimulation but vary considerably in their sensitivity to beta/gamma heterodimers. For example, in the presence of activated G_s -alpha the type II and IV adenylyl cyclases are stimulated by beta/gamma released upon receptor activation,

while the type I enzyme is inhibited by beta/gamma. Other adenylyl cyclase isozymes are unaffected by beta/gamma. Regulation of adenylyl cyclase activity is also controlled by intracellular Ca^{2+} levels; the type I and III adenylyl cyclases are stimulated by calmodulin (CaM) and Ca^{2+} (Krupinski et al. 1989; Choi et al. 1992a) whereas the other four enzymes are insensitive to CaM.

The m4 muscarinic receptor regulates intracellular cAMP through a number of discrete G protein pathways. In general, the activation of m2 and m4 receptors are thought to directly inhibit cAMP production via G_i while the m1, m3, and m5 receptors couple to stimulation of phosphoinositide (PI) hydrolysis (Peralta et al. 1988; Ashkenazi et al. 1989a). Muscarinic receptor stimulated PI turnover increases intracellular Ca^{2+} which can activate the type I adenylyl cyclase (Choi et al. 1992a). In certain cell types, m4 receptors can also couple weakly to PI hydrolysis suggesting that m4 activation might also be able to stimulate adenylyl cyclase activity in tissues expressing Ca^{2+} /CaM sensitive isozymes (Ashkenazi et al. 1989b). The recent demonstration that G_i coupled receptors can regulate adenylyl cyclase activity via beta/gamma subunits has suggested a third mechanism by which m4 receptors might regulate intracellular cAMP levels (Federman et al. 1992). In fact, beta/gamma activation of specific adenylyl cyclase isozymes might explain the recent data indicating that m4 activation can stimulate adenylyl cyclase in a Ca^{2+} -independent manner in the olfactory bulb and certain cultured cell lines (Olianas and Onali 1990, 1991; Baumgold et al. 1992. Jones et al. 1991).

Since m4 receptor activation can either inhibit and/or stimulate cAMP production in different tissues, we hypothesized that m4 control of cAMP might be determined by differential expression of adenylyl cyclase isozymes. In this study, we expressed the m4 receptor in HEK-293 cells expressing the two known Ca^{2+} /CaM sensitive adenylyl cyclases, type I and III. We were particularly interested in the CaM sensitive adenylyl cyclases because they are differentially regulated by a number of distinct pathways including, G_s -alpha stimulation, G_i -alpha inhibition, intracellular Ca^{2+} levels, and beta/gamma subunits (reviewed in Choi et al. 1993a).

In this chapter I demonstrate that m4 muscarinic receptors can couple to either stimulation or inhibition of the type I and type III adenylyl cyclases through G protein mediated processes. The data in this chapter is consistent with "cross-over" of m4 receptors from G_i inhibition to G_s stimulation of adenylyl cyclase activity.

Methods

Cell culture and DNA transfection

Human embryonic kidney 293 cells were grown at 36° C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum in a humidified 95 % air/5 % CO₂ incubator. Cell culture materials were from Gibco unless otherwise noted. Neomycin-resistant HEK-293 cells stably transfected with an expression vector CDM8 that contained cDNA for type I adenylyl cyclase (CDM8(I-AC)), type III adenylyl cyclase (CDM8(III-AC)), or no exogenous DNA were used for this study. Particular clones of each type (1AC43, 3AC3, and 293-NEO) have been previously characterized (Choi et al. 1992b, 1993b) and were used for subsequent co-transfection with m4 muscarinic receptors. Each of these cell lines were stably transfected with either pCD-PS expression vector containing DNA encoding the chick m4 muscarinic receptor or pCD-PS vector alone (Bonner et al. 1988). Briefly, cells were plated in 100-mm dishes at a density of 2×10^6 cells/ plate, grown overnight and transfected with the pCD control vector or pCD(m4) (10 µg DNA/plate) and a hygromycin resistance vector (1 µg DNA/plate) by the calcium phosphate method (Chen & Okayama 1987). Hygromycin-resistant cells were selected in culture medium containing Hygromycin B (Sigma, 460 Units/ml) and 300 µg/ml G418. Clones of hygromycin/ neomycin-resistant cells were examined for [³H] QNB binding to detect cells that expressed m4 muscarinic receptor. M4-expressing cells were also examined for adenylyl cyclase activity (via forskolin and/or Ca²⁺/Cam stimulation) to ensure that CaM sensitive adenylyl cyclase activities were maintained and that the level of expressed adenylyl cyclase was the same between cell lines expressing varying levels of m4 receptors. After selection, cells were maintained in media containing 230 U/ml hygromycin B and 300 µg/ml G418. Ten hygromycin/neomycin resistant clones expressing varying levels of m4 receptor and each adenylyl cyclase type were isolated and characterized further. Four hygromycin/neomycin resistant clones transfected with control vector pCD and each adenylyl cyclase were isolated and characterized. Cell line nomenclature is as follows: Cells transfected with pCD control vector were designated "P", cells transfected with m4-pCD were designated "M", cells expressing type I or III adenylyl cyclase were designated "1" or "3" respectively, neomycin resistant cells transfected with control CDM8 vector were designated "N". Thus, for example, neomycin/ hygromycin-resistant cells expressing m4 receptors and no exogenous

adenylyl cyclase were designated "MN" followed by an arbitrary clone number (e.g. MN-15).

³H] QNB binding assays

Expression levels of m4 muscarinic receptors were determined by measuring specific [³H] QNB binding to cell membrane homogenates as described by Nathanson et al. (1992). Briefly, cells were grown to confluence in 100 mm culture dishes, washed twice with ice-cold PBS, and harvested in 4 ml ice-cold 50.0 mM sodium phosphate, pH 7.4. Cells were homogenized by hand with 20 strokes of a ground glass homogenizer and centrifuged at 30,000 x g, 4° C, for 15 minutes. The pellets were washed once in NaPO₄, recentrifuged, and resuspended in 0.5 ml of 50 mM NaPO₄. Duplicate samples of 0.1 ml of resuspended membranes were incubated for 90 min at 25 ° C with 4.2 x 10⁻¹⁰ M [³H] QNB (Amersham) in a final volume of 1.0 ml. Nonspecific binding was measured in parallel in the presence of 1.0 uM atropine.

Cyclic AMP accumulation.

Changes in intracellular cAMP levels were measured by determining the ratio of [³H] cAMP to a total ATP and ADP pool in [³H] adenine loaded cells as described by Wong et al. (1991). Confluent cells in 6-well plates were initially incubated in DMEM containing [³H] adenine (2.0 µCi/ml, ICN) for 16-20 hours, washed once with 150 mM NaCl, and incubated at 37° C for 30 min in incubation buffer (118 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 0.5 mM EDTA, 10.0 mM glucose, 20.0 mM HEPES, pH 7.4) containing 1.0 mM IBMX, 5.0 µM forskolin and various effectors as indicated. Reactions were terminated by aspiration, washing cells once with 150 mM NaCl, and adding 1.0 ml of ice-cold 5% TCA containing 1.0 µM cAMP. Culture dishes were maintained at 4° C for 1-4 hours and acid-soluble nucleotides were separated by ion-exchange chromatography as described (Salomon et al. 1974).

While this assay system affords rapid and extremely sensitive measurements of relative changes in intracellular cAMP levels in response to carbachol, we were initially concerned about reproducibility because we, like others (Federman et al. 1992), noticed that absolute numbers for cAMP accumulation often varied between experiments. It is important to emphasize that relative changes in cAMP were highly consistent between experiments. However, to ensure that these changes in cAMP accumulation were accurate and to assess the relative contribution of transfected adenylyl cyclase vs. endogenous

adenylyl cyclases expressed in these cells, we used the more laborious method of Gilman (1970) to measure absolute cAMP levels in several cell lines expressing type I, type III, or no exogenous adenylyl cyclase. These results confirmed the relative changes in cAMP levels previously observed in response to carbachol in all cell lines tested. For example, treating forskolin stimulated type III adenylyl cyclase expressing cell lines P3-2, M3-14, and M3-32 with 1.0 mM carbachol altered intracellular cAMP levels (pmol cAMP formed/mg protein) from 4649.9 ± 346.2 to 4677.9 ± 29.4 ; 4762.0 ± 335 to 2229.6 ± 27.6 ; and 4268.0 ± 29.4 to 7410.3 ± 282.5 , respectively. Furthermore, these experiments demonstrated that relative changes in cAMP in Type I and III adenylyl cyclase cell lines were primarily due to regulation of these exogenously expressed adenylyl cyclase. For example, forskolin stimulated levels of cAMP in control HEK-293 cells (PN-5) were only 551.8 ± 99.5 pmol/mg compared to 3209.1 ± 13.5 and 4649.9 ± 346.2 pmol/mg in Type I (P1-6) and III (P3-2) expressing cell lines, respectively. These results indicated that endogenous adenylyl cyclases contributed at most only 10-20 % to the relative changes in cAMP levels observed in forskolin-stimulated type I and III expressing cells.

Cell membrane preparation

Membranes were prepared as described in Choi et al. (1992a, b). Briefly, confluent cells were washed twice with 150 mM NaCl, harvested into ice-cold membrane buffer (20 mM Tris-HCl, pH 7.4, 2.0 mM MgCl₂, 1.0 mM EDTA, 0.5 mM dithiothreitol, 5.0 µg/ml leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride), and broken at 4° C by Dounce homogenization. Membranes were separated from nuclei and unbroken cells by centrifugation at 600xg for 2 min and the supernatants were subjected to centrifugation at 30,000 g for 20 min. The resulting membrane pellets were resuspended in membrane buffer (4° C) to a typical protein concentration of 0.2 to 0.8 mg/ml and assayed for adenylyl cyclase activity. Protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical).

Adenylyl cyclase assays

Adenylyl cyclase activity was determined by adding membrane fractions (10-40 µg protein) to an assay solution containing 1.0 mM alpha [³²P]-ATP (500 cpm/pmol), [³H]-cyclic AMP (20,000 cpm/µmol), 5.0 mM MgCl₂, 0.2 mM EGTA, 1.0 mM EDTA, 2.0 mM cyclic AMP, 1.0 mM beta-mercaptoethanol, 5.0 mM theophylline, 0.1 % bovine serum albumin, 20 mM phosphocreatine, and 100 units/ml creatine phosphokinase in 20

mM Tris-HCl, pH 7.4 in a final volume of 250 μ l. After 25 min at 30° C, reactions were terminated by adding 750 μ l 1.5% SDS and [³²P]-cyclic AMP generated was measured as described by Salomon et al. (1974). Peptide m4:393-411 (RSQVRKKRQMAAREKKVTR) was synthesized and purified by the University of Washington Molecular Pharmacology Protein Core facility.

Quantitation of intracellular free Ca²⁺

Cell lines grown on glass cover slips were examined for intracellular free Ca²⁺ using fura-2 by the method of Hassid (1986).

Results

To examine the coupling of m4 muscarinic receptors to type I and III adenylyl cyclases, human embryonic kidney (HEK-293) cells stably expressing either type I or type III adenylyl cyclase were transfected with plasmids containing the m4 muscarinic receptor DNA and a gene conferring resistance to the antibiotic hygromycin. HEK-293 cells were chosen because they contain low levels of endogenous adenylyl cyclases and the regulation of type I and III adenylyl cyclases in these stable lines has been characterized (Choi et al. 1992b, 1993b). Forty independent hygromycin resistant cell lines of each adenylyl cyclase type were screened for stable m4 expression by [³H] QNB binding and several lines expressing varying levels of m4 receptor were maintained for subsequent studies (Figure 12). Control cell lines transfected with hygromycin resistance and plasmid lacking m4 were also selected. All cell lines were further screened for type specific adenylyl cyclase activity (i.e. Ca²⁺/Cam sensitivity) to ensure adenylyl cyclase expressions were maintained .

Wild type HEK-293 cells apparently express low levels of endogenous m1 or m3 muscarinic receptors since carbachol treatment of these cells expressing type I adenylyl cyclase indirectly stimulated cAMP production by increasing intracellular free Ca²⁺ (Choi et al. 1992b). However, no specific QNB binding in wild type cells or hygromycin resistant control cell lines was detectable indicating that endogenous muscarinic receptor levels are very low (Figure 12). In cell lines expressing type I or type III adenylyl cyclase, m4 receptor expression varied from 25 to 400 fmol [³H] QNB bound/mg protein (Figures 12a, b). Control cells expressing no exogenous adenylyl cyclases had receptor levels ranging from 50 to 450 fmol [³H] QNB bound/mg protein (Figure 12c). These

levels of receptor expression are consistent with m4 levels in several brain tissues (Wall et al. 1992 a, b).

We initially examined cells expressing different levels of m4 receptor with or without the type I or III adenylyl cyclases by monitoring changes in intracellular cAMP production after exposure to the muscarinic agonist, carbachol. Carbachol treatment of cell lines expressing type I adenylyl cyclase but no exogenous m4 receptor (e.g. P1-6, Figure 2a) caused a small increase in cAMP production with an EC₅₀ of approximately 50 μ M. These data are consistent with the results of Choi et al. (1992b) who demonstrated a Ca²⁺-dependent carbachol stimulation of the type I adenylyl cyclase expressed in HEK-293 cells, presumably via IP₃ mediated Ca²⁺ increases. Intracellular cAMP levels declined in response to carbachol in most cell lines expressing type I adenylyl cyclase with low to moderate levels of the m4 receptor (e.g. M1-32, M1-37), presumably via m4 activation of G_i (Figure 13a). Maximal inhibition for each cell line in the presence of 1.0 mM carbachol varied from 40 % (cell line M1-37) to 80 % (cell line m1-33) (i.e. 20-60 % of cAMP accumulation in the absence of carbachol). In cell line M1-36, which expressed type I adenylyl cyclase and relatively higher levels of m4, carbachol stimulated cAMP levels 1.5 to 2.5 fold (Figure 13a).

Cell lines expressing the type III adenylyl cyclase and varying levels of m4 receptor showed similar responses to carbachol (Figure 13b). Carbachol treatment of control cells expressing no exogenous muscarinic receptor had no effect on cAMP production (e.g. cell line P3-2, Figure 13b). These results are consistent with our previous findings which indicated that endogenous muscarinic receptors in HEK-293 cells do not affect type III adenylyl cyclase activity (Choi et al. 1992a, b). Like type I adenylyl cyclase containing cells, cAMP production was inhibited approximately 50 % by carbachol in type III cell lines expressing low to moderate levels of m4 receptors (e.g. M3-37, M3-14, Figure 13b). However, in cell line M3-32, which expressed the highest levels of m4, carbachol treatment increased cAMP production approximately 1.5 fold. Maximal stimulation of cAMP production in M3-32 was generally slightly lower than M1-36, varying from 120 to 200% of control levels in 4 experiments performed.

Cyclic AMP levels in wild type HEK-293 cells expressing no exogenous adenylyl cyclase and no exogenous muscarinic receptor were unaffected by carbachol treatment (e.g. PN-5, Figure 13c). In m4 expressing lines, cAMP accumulation was inhibited approximately 40-60 % by carbachol in a dose-dependent manner (e.g. MN-5, Figure

13c). In all cell lines, 1.0 μM atropine, a muscarinic antagonist, blocked the effects of 1.0 mM carbachol on cAMP production.

The concentration dependence for carbachol effects on intracellular cAMP in M1-36 and M3-32 cells was examined in greater detail over an expanded range of carbachol concentrations (Figure 14). Interestingly, treatment of both M1-36 and M3-32 with low carbachol concentrations decreased cAMP production to 50 to 60 % while higher concentrations of carbachol stimulated cAMP levels approximately 1.5 fold. In both cell lines, maximal cAMP inhibition was observed at 0.1 to 0.5 μM carbachol with an EC_{50} of approximately .01 μM while the EC_{50} for stimulation ($\sim 5.0 \mu\text{M}$) was approximately 100 fold higher. In comparison, the EC_{50} for carbachol inhibition in cell lines that only demonstrated cAMP inhibition (e.g. M1-37, M3-14, M3-37) ranged between 1.0 to 10.0 μM carbachol (Figure 13). Atropine treatment blocked both the carbachol mediated inhibition and stimulation of cAMP accumulation in these cells although at high agonist concentrations, cAMP levels were slightly lowered.

In most m4 expressing cell lines, we observed classical m4 receptor-mediated inhibition of cAMP, presumably via G_i activation. We were particularly interested, however, in understanding the mechanisms by which m4 receptors stimulated the accumulation of cAMP in cell lines M1-36 and M3-32. Two primary hypothesis have been proposed to explain muscarinic receptor stimulation of adenylyl cyclases. The first suggests that muscarinic stimulation of PI turnover and subsequent increases in intracellular Ca^{2+} activate Ca^{2+} /Cam sensitive adenylyl cyclases (Felder et al. 1989; Jansson et al. 1991; Choi et al. 1992b). The second hypothesis proposes direct activation of beta/gamma sensitive isozymes of adenylyl cyclase (Federman et al. 1992; Baumgold 1992). Since the type I and III adenylyl cyclases are not stimulated by beta/gamma (Tang and Gilman 1991), we investigated the role of Ca^{2+} in m4 mediated stimulation of cAMP in cell lines M1-36 and M3-32.

To determine the role of Ca^{2+} in carbachol stimulation of intracellular cAMP, we preloaded cells with the intracellular Ca^{2+} chelator BAPTA/AM to block any carbachol stimulated increases in $[\text{Ca}^{2+}]_i$. Preliminary experiments using fura-2 loaded M1-36 and M3-32 cells indicated that treatment with 0.5 mM carbachol elicited a rapid 4-5 fold increase in $[\text{Ca}^{2+}]_i$ from approximately 40 to 160 μM . Pre-treatment of these same cells with 100.0 μM BAPTA/AM for 30 min completely buffered any Ca^{2+} increases during a 30 min exposure to 0.5 mM carbachol (data not shown). We then examined the effect of BAPTA/AM treatment on carbachol stimulated cAMP increases. In both cell lines,

carbachol inhibition at low concentrations and stimulation at high concentrations were unaffected by BAPTA buffering of $[Ca^{2+}]_i$ suggesting that Ca^{2+} was not required for any of the carbachol mediated effects on cAMP accumulation (Figure 14a,b).

To further characterize the role of Ca^{2+} in m4 stimulation of adenylyl cyclase, we directly measured adenylyl cyclase activity in membranes in the absence of Ca^{2+} (Figure 15). Because m4 coupled G_i -mediated inhibition of adenylyl cyclase in isolated membranes requires high NaCl concentrations (Lichtshtein et al. 1979), we performed assays both in the presence and absence of NaCl. Since we were primarily interested in the mechanism of m4 stimulation of adenylyl cyclase, we initially performed assays in the absence of NaCl to eliminate inhibitory coupling. In the presence of 10.0 μ M GTP, 5.0 μ M forskolin, 200 μ M EGTA and no added Ca^{2+} or NaCl, carbachol did not inhibit adenylyl cyclase activities in membranes isolated from any cell line tested (Figure 15). Under these conditions, type I adenylyl cyclase activity in M1-36 membranes was stimulated approximately 1.5 fold by carbachol with a concentration dependence similar to that in whole cells ($EC_{50} = 5-10 \mu$ M). In the absence of NaCl, adenylyl cyclase activity in membranes isolated from cell line M1-37, which exhibited only carbachol mediated cAMP inhibition in whole cell experiments, was also stimulated in a dose-dependent manner by carbachol. In type III adenylyl cyclase expressing cell lines, only membranes isolated from cell line M3-32 demonstrated carbachol stimulation of adenylyl cyclase activity (data not shown). In all cases, 1.0 μ M atropine blocked carbachol stimulation of adenylyl cyclase activity. In the presence of high NaCl (100mM), carbachol stimulation of adenylyl cyclase activity in cell lines M1-37 and M1-36 was reduced 66 and 75 %, respectively, presumably due to competing inhibitory actions of G_i (data not shown). In cell lines, expressing lower m4 receptor levels (e.g. M3-14), 1.0 mM carbachol inhibited adenylyl cyclase activity in membranes 20-30 % in the presence of 100 mM NaCl. The addition of low concentrations of forskolin was required to observe m4-mediated inhibition of adenylyl cyclase activity (e.g. Lichtshtein et al. 1979) and improved the ability of m4 to stimulate adenylyl cyclase.

Since m4 stimulation of either type I or III adenylyl cyclase did not require Ca^{2+} , we wondered if this stimulatory coupling was dependent on activation of a G protein. To examine this question, we measured carbachol regulation of adenylyl cyclase activity in membranes isolated from cell line M1-36 in the presence or absence of GDP-beta-S, a GDP analogue which blocks G protein activation. In the presence of 10.0 μ M GTP, 50.0

μM GDP-beta-S completely inhibited carbachol mediated stimulation of type I adenylyl cyclase but basal adenylyl cyclase activity was unaffected (Figure 16).

Since m_4 receptors are known to couple to G_i and G protein activation was apparently required for carbachol stimulation of adenylyl cyclase in these cells, we evaluated the role of G_i for m_4 stimulation using pertussis toxin (PTX). We also anticipated that inhibition of G_i activity with PTX might unmask G_s coupling to m_4 receptors that is not normally seen when G_i is functional. PTX treatment of cell line M1-36, in which cAMP accumulation was inhibited at low carbachol concentrations and stimulated at high carbachol concentrations, completely eliminated carbachol inhibition of cAMP and revealed a dramatic increase in cAMP accumulation in response to carbachol (Figure 17a). Toxin treatment did not shift the apparent EC_{50} for carbachol stimulation, however cAMP production was stimulated 10-fold over basal levels. Interestingly, in cell line M1-37, which exhibited only cAMP inhibition in response to carbachol, PTX treatment abolished cAMP inhibition and unmasked a two-fold carbachol mediated stimulation of cAMP. Similar results were seen in other m_4 /type I adenylyl cyclase expressing cell lines that were examined. cAMP production in type I adenylyl cyclase expressing cells, which expressed no exogenous muscarinic receptor, was not affected by PTX treatment (data not shown).

In all type III adenylyl cyclase cell lines, PTX treatment blocked m_4 mediated inhibition of cAMP accumulation. Carbachol stimulation of cAMP in M3-32 cells was increased from two to seven fold by PTX treatment (Figure 17b). As in type I adenylyl cyclase cell lines, PTX treatment of type III cell lines inhibited by carbachol, uncovered a carbachol mediated stimulation of cAMP at higher agonist concentrations (e.g. M3-14, Figure 17b). PTX treatment of type III adenylyl cyclase cell lines which expressed no exogenous m_4 receptors did not affect cAMP metabolism. To ensure that the increased cAMP stimulation caused by PTX was still not due to increases in $[\text{Ca}^{2+}]_i$, we preloaded PTX treated M1-36 cells with BAPTA/AM prior to carbachol exposure and determined that this increased cAMP accumulation was unaffected by Ca^{2+} chelation (Figure 17a).

Because pertussis toxin treatment of cells uncoupled G_i -mediated inhibition and revealed a dramatic increase in cAMP accumulation, we wondered if the differential coupling by m_4 receptors to inhibition and stimulation of adenylyl cyclase might be due to different affinities of the m_4 receptor for G_i and G_s . Recently, Okamoto and Nishimoto (1992) described a polypeptide sequence within the human m_4 muscarinic receptor which apparently represents the G_i activating region for this receptor. Synthetic peptides

corresponding to residues 375-400 of the human m4 receptor were able to directly activate G_i at nanomolar concentrations in a magnesium dependent manner (Okamoto and Nishimoto 1992). Interestingly, this peptide was also able to activate G_s but at 100-fold higher concentrations. Since stimulation of adenylyl cyclase by the m4 receptor required approximately 100-fold higher concentrations of carbachol than inhibition (Figure 14), we tested whether a peptide corresponding to this region in the chick m4 receptor (M4: 393-411) could directly mimic the differential effects of the activated receptor on adenylyl cyclase activity. Using membranes isolated from cell line P3-2, which stably expressed the type III adenylyl cyclase and no exogenous muscarinic receptor, we measured adenylyl cyclase activity in response to increasing concentrations of peptide m4:393-411. Initial experiments with forskolin-stimulated activity indicated that m4:393-411 elicited a biphasic response similar to that observed with agonist-activated m4 receptor: slight inhibition (10%) at low peptide concentrations (1.0 μM) and increasing adenylyl cyclase activity at higher concentrations ($\sim 50 \mu\text{M}$). As with intact m4 receptor, the inhibitory phase of this response was only observed in the presence of 100mM NaCl. To more carefully examine peptide stimulation of adenylyl cyclase, we treated P3-2 cells with PTX to eliminate potential G_i -mediated competing effects and measured adenylyl cyclase activity in isolated membranes with increasing concentrations of m4:393-411 (Figure 18). In PTX-treated membranes, M4:393-411 did not inhibit adenylyl cyclase activity at any concentration but stimulated adenylyl cyclase activity 1.6 fold with an EC_{50} of 50 μM . At high peptide concentrations ($\geq 100 \mu\text{M}$), stimulation of adenylyl cyclase activity was reduced. These results are consistent with the cross-over of m4 receptor G protein activating peptides from G_i to G_s activation observed by Okamoto and Nishimoto (1992) and suggest that stimulation of adenylyl cyclase by m4 receptors occurs via G_s activation.

If m4 receptor activation of G_s is responsible for adenylyl cyclase stimulation, one might expect that cholera toxin pre-treatment of M1-36 or M3-32 cells would reduce the available pool of G_s which can couple to m4 and reduce the level of adenylyl cyclase stimulation at high carbachol concentrations. Indeed, pre-treatment of M1-36 cells overnight with cholera toxin decreased the carbachol-mediated stimulation of adenylyl cyclase activity (data not shown). These results are difficult to interpret, however, because cholera toxin treatment may directly and maximally activate G_s -mediated adenylyl cyclase activity.

Discussion

Jones et al. (1991) have shown that m4 receptors in transfected CHO cells display a biphasic response; at low agonist concentrations cAMP accumulation is inhibited while at higher agonist concentrations cAMP accumulation is stimulated. The mechanism underlying this Ca^{2+} -independent m4 stimulation of adenylyl cyclase is not known, although it has been hypothesized that this phenomenon may be due to stimulation of beta/gamma sensitive adenylyl cyclase isozymes (Federman et al. 1992; Baumgold 1992; Bourne and Nicoll 1993). It has been difficult to define the role of beta/gamma in this phenomenon because coupling of m4 receptors to specific forms of adenylyl cyclase with known sensitivities to beta/gamma had not been examined until this study.

We have discovered that m4 receptors can couple to inhibition or stimulation of CaM sensitive adenylyl cyclases. Inhibition of the type I and III adenylyl cyclase activities was most likely mediated via G_i activation since PTX treatment completely eliminated inhibition. While inhibition of the type I enzyme might have been due to beta/gamma inhibition, the carbachol concentration dependence for inhibition of the type I and III adenylyl cyclases was similar. Furthermore, beta/gamma regulation could not explain inhibition of the type III adenylyl cyclase since it is not affected by beta/gamma. In isolated cell membranes, inhibition of adenylyl cyclase was only apparent in the presence of high NaCl concentrations. This NaCl requirement for G_i coupling may explain the seemingly paradoxical stimulation of adenylyl cyclase in membranes isolated from cell line M1-37, which exhibited cAMP inhibition in intact cells. In the absence of NaCl, G_i -mediated inhibition is uncoupled and competing stimulatory responses may be revealed.

While in most cell lines m4 activation inhibited cAMP accumulation, in cell lines expressing the highest levels of m4, carbachol elicited a biphasic response. The stimulatory component was apparent in all cell lines after PTX treatment suggesting that there may be competition between PTX-sensitive and insensitive G proteins for receptor recognition. The type I enzyme appeared to be slightly more sensitive to carbachol stimulation compared to the type III adenylyl cyclase but this may reflect higher levels of m4 expression in type I expressing cells.

What is the mechanism underlying m4-mediated increases in cAMP? Experiments with BAPTA treated cells and adenylyl cyclase assays in isolated membranes demonstrated that stimulation of the type I or III adenylyl cyclases by m4 receptors did not require Ca^{2+} . These results are consistent with those reported in the olfactory bulb, where both the type I and III adenylyl cyclases are expressed (Xia et al. 1991; Glatt and

Snyder 1993), but differ from results in SK-N-SH cells where Ca^{2+} -independent muscarinic stimulation of adenylyl cyclase was only apparent in whole cells (Baumgold et al. 1992). In m4-transfected CHO cells, m4 induced increases in cAMP with a carbachol concentration dependence similar to what we observed, but the mechanism for stimulation in this system was not determined and the types of adenylyl cyclases expressed in these cells is unknown (Jones et al. 1991).

Several lines of evidence indicate that the stimulation of type I and III adenylyl cyclases by m4 receptors was not due to m4-mediated release of beta/gamma from G_i or G_o . Beta/gamma stimulation seems unlikely since the type I adenylyl cyclase is inhibited and the type III enzyme is unaffected by beta/gamma subunits (Tang and Gilman 1991; Taussig et al. 1993). Furthermore, PTX treatment of these cells enhanced the m4 stimulation of adenylyl cyclase rather than blocking stimulation as would be expected if beta/gamma released from G_i or G_o was responsible. Stimulation of the type I and III adenylyl cyclases in membranes required GTP suggesting that stimulation was mediated via a stimulatory G protein, presumably G_s .

Recently, Nishimoto and his colleagues described a polypeptide sequence motif within several G protein coupled receptors which apparently represents specific G protein activator regions (Okamoto et al. 1991; Okamoto and Nishimoto 1992; Ikezu et al. 1992). Within the human m4 receptor, amino acids sequences 130-147, 217-240, and 375-400 are potential G protein interaction domains and peptides corresponding to these sequences stimulate GTP binding to specific G proteins. Synthetic peptides corresponding to residues 130-147 and 375-400 were able to activate G_i/G_o but only peptide 375-400 could activate at nanomolar concentrations (Okamoto and Nishimoto 1992). Since the activation domain corresponding to residues 130-147 is conserved across all known muscarinic receptors and activator region 375-400 only occurs in m2 and m4 receptors, the 375-400 sequence is most likely the G_i/G_o activation site (Okamoto and Nishimoto 1992). Interestingly, peptides 130-147 and 375-400 were also able to activate G_s but at concentrations 30-100 times greater than required for G_i activation (Okamoto and Nishimoto 1992). In the chick m4 receptor, which was used in this study, all three G protein activator regions present in human m4 are conserved (Tietje et al. 1990). Peptides derived from two of these domains had significant inhibitory and stimulatory effects on adenylyl cyclase activity while the third had no effect (Dittman and Storm, unpublished observation). Peptide m4:393-411 (corresponds to peptide 375-400 in the human m4 receptor) was able to mimic the dose-dependent biphasic inhibitory and stimulatory actions

of activated m4 receptor on adenylyl cyclase activity. Therefore, the ability of m4 receptors to either inhibit or stimulate CaM sensitive adenylyl cyclases may reflect the differential affinities of these domains for G_i vs G_s . Since the affinity of these domains are apparently higher for G_i than G_s , m4 stimulation of adenylyl cyclases via G_s may only be evident when m4 receptor density is high, or when G_i is inhibited with PTX. It is interesting to note that the stimulation by the M4 peptide was lower than with M4 agonist under the same conditions, presumably because of the lower efficacy of an added peptide compared to the holo-receptor expressed in the plasma membrane.

The human α_2C10 adrenergic receptor also contains several putative G protein activator regions including residues 131-148 in the second cytoplasmic loop which specifically activates G_s with a similar EC_{50} for G_s activation as domain 375-400 of m4 (Okamoto and Nishimoto 1992). It is interesting that this adrenergic receptor subtype can also functionally couple to both G_i -mediated inhibition and G_s -mediated stimulation of adenylyl cyclase (Fraser et al. 1989; Eason et al. 1992).

What role does m4 receptor coupling to G_s play in cellular signal transduction? In the olfactory bulb and retina (Olianas and Onali 1990, 1991; Brown and Rietow 1982), Ca^{2+} -independent stimulation of adenylyl cyclase by muscarinic receptors has been reported, although stimulation is apparently sensitive to PTX in the olfactory bulb (Olianas and Onali 1992). M4 activation of G_s may act directly to stimulate adenylyl cyclases or alternatively the dual G_s and G_i coupling properties of m4 might facilitate beta/gamma stimulation of type II and IV adenylyl cyclases. Since activated G_s -alpha is required for beta/gamma stimulation of type II and IV adenylyl cyclase, receptors such as m4 or α_2C10 , which can activate both G_s -alpha and stimulate beta/gamma release from G_i or G_o , may provide signal integration through a single receptor (Tang and Gilman 1991; Bourne and Nicoll 1993).

In summary, we have demonstrated that the m4 muscarinic acetylcholine receptors can couple to G_i -mediated inhibition or G_s -mediated stimulation of CaM sensitive adenylyl cyclases, depending upon receptor density. These results and the recent demonstration of G_s activating domains in m4 and α_2 adrenergic receptors suggests that the m4 receptors may belong to a subclass of dual functioning receptors that can show cross-over from G_i - to G_s -mediated regulation of adenylyl cyclases. We can now define four coupling mechanisms for muscarinic receptor regulation of adenylyl cyclases which include direct inhibition through G_i , indirect stimulation of CaM sensitive enzymes by mobilization of intracellular Ca^{2+} , modulation of adenylyl cyclases by released

beta/gamma, and direct stimulation through Gs coupling. All of these mechanisms may play important regulatory roles in specific areas of the nervous system.

FIGURE 12. Levels of stable m4 muscarinic receptor expression in 293 HEK cells expressing Ca^{2+} /Cam sensitive adenylyl cyclases. Stable cells line expressing both m4 muscarinic receptors and exogenous adenylyl cyclases were generated by co-transfecting the m4-pCD vector and a hygromycin B resistance vector into stable lines expressing type I or type III adenylyl cyclase, or neomycin resistance alone. M4 receptor expression levels were determined by measuring [3H] QNB binding to membrane homogenates as described in experimental procedures. Specific binding was determined by subtracting amount bound in the presence of 1.0 μM atropine. Cell line nomenclature is as follows: Cells transfected with pCD control vector were designated "P", cells transfected with m4-pCD were designated "M", cells expressing type I or III adenylyl cyclase were designated "1" or "3" respectively, neomycin resistant cells transfected with control CDM8 vector were designated "N". Thus, for example, neomycin/ hygromycin- resistant cells expressing m4 receptors and no exogenous adenylyl cyclase were designated "MN" followed by an arbitrary clone number (e.g. MN-15). A) Distinct cell lines expressing type 1 adenylyl cyclase and varying levels of m4 muscarinic receptor. B) Distinct cell lines expressing type III adenylyl cyclase and varying levels of m4 muscarinic receptor. C) HEK-293 cells expressing no exogenous adenylyl cyclase and varying levels of m4 muscarinic receptor.

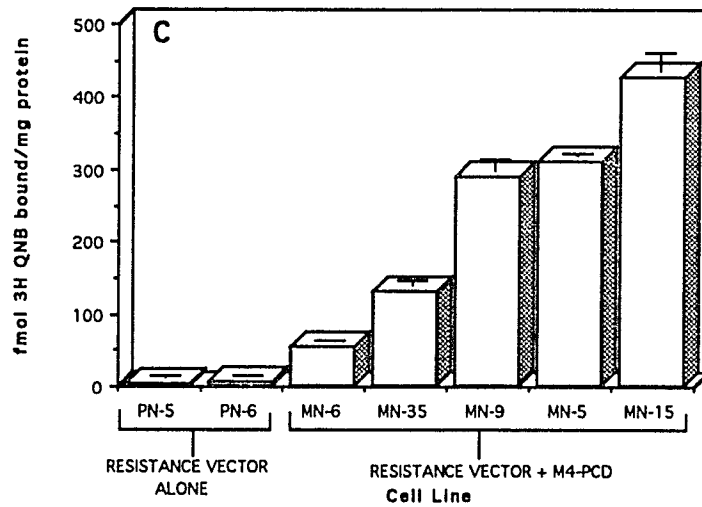
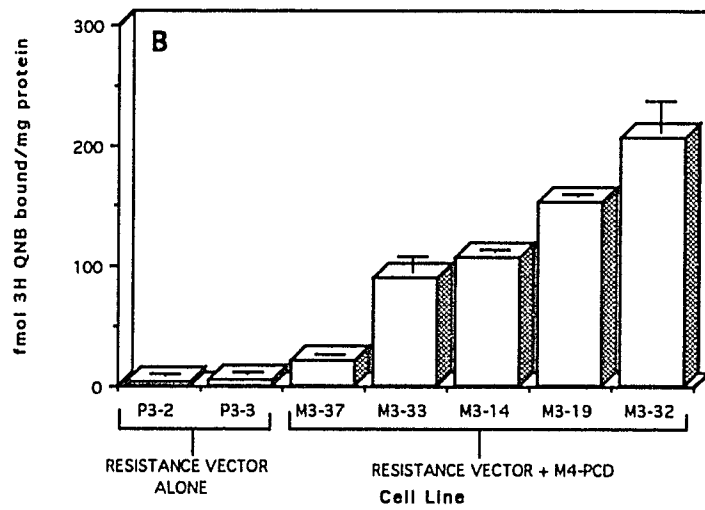
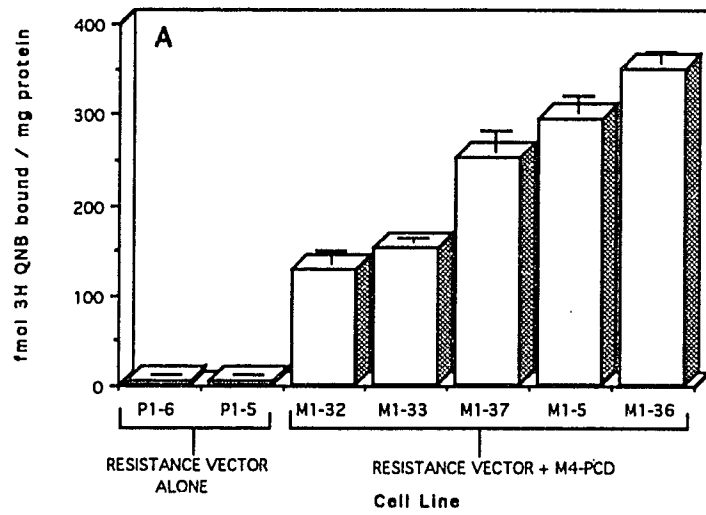


FIGURE 13. Effect of carbachol on cAMP accumulation in representative HEK-293 cells expressing Ca^{2+} /Cam sensitive adenylyl cyclases and varying levels of m4 muscarinic receptors. Cell lines stably expressing varying levels of m4 receptor and A) type I adenylyl cyclase [cell line P1-6 O (no m4); M1-32 ▲ (Low m4); M1-37 □ (Med. m4); M1-36 ● (High m4)], B) type III adenylyl cyclase [cell line P3-2 O (no m4); M3-37 ▲ (Low m4); M3-14 □ (Med. m4); M3-32 ● (High m4)] or C) neomycin resistance alone [cell line PN-5 O (No m4); MN-5 ▲ (High m4)] were exposed for 30 min to incubation buffer containing 5.0 μM Forskolin, 1.0 mM IBMX and increasing concentrations of the muscarinic agonist carbachol. To ensure the effects of carbachol were mediated via muscarinic activation, each cell line was also treated with the highest carbachol concentration (1.0 mM) in the presence of 1.0 μM atropine. Cyclic AMP accumulations were determined as described in Experimental Procedures. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation for each cell line in the absence of carbachol. These data represent triplicate determinations in one experiment; three additional experiments gave similar results. Taken together, data from four independent experiments indicated that there is no significant difference in the dose-dependence of cAMP inhibition between cell lines M1-32 and M1-37 as suggested by the representative data presented in Figure 13A.

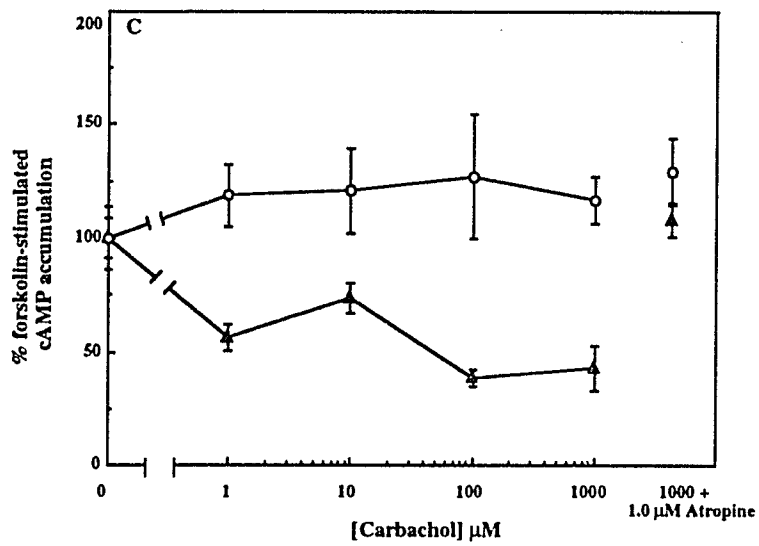
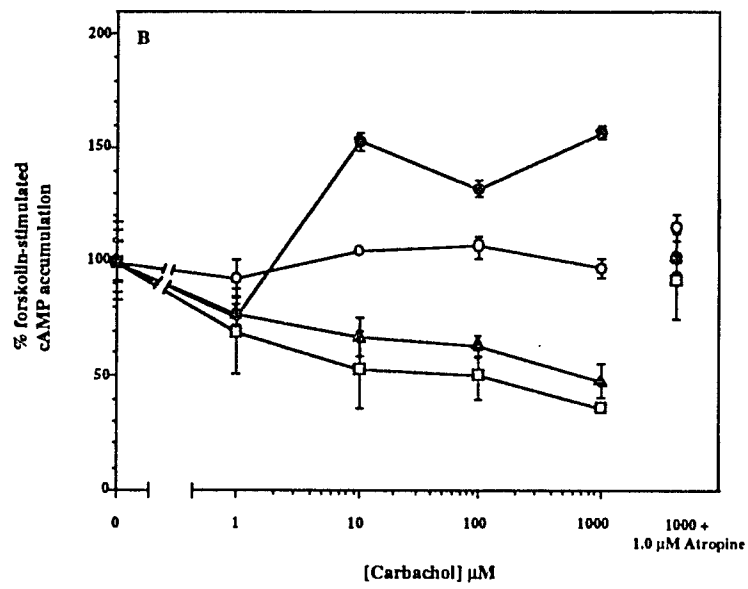
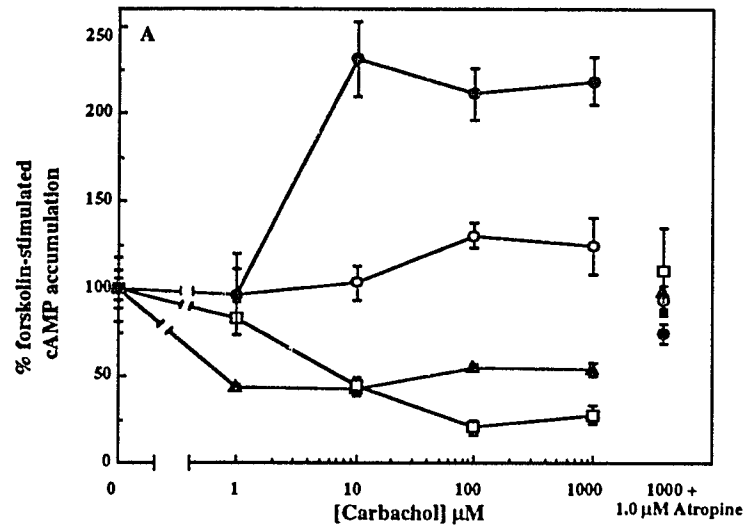


FIGURE 14. Effects of BAPTA/AM on carbachol-mediated increases in cAMP accumulation in HEK-293 cells expressing Ca^{2+} /Cam sensitive adenylyl cyclases and m4 muscarinic receptors. Cell lines expressing m4 receptors and either A) type I adenylyl cyclase (M1-36) or B) type III adenylyl cyclase (M3-32) were exposed to carbachol in the presence (Δ) or absence (\blacktriangle , O) of 1.0 μM atropine for 30 min. in incubation buffer containing 5.0 μM Forskolin and 1.0 mM IBMX. Relative cAMP accumulations were determined as described in Experimental Procedures. Immediately prior to carbachol exposure, cells were incubated for 30 min. in the presence (\blacktriangle) or absence (Δ ,O) of 100.0 μM BAPTA/AM in non-supplemented DMEM and washed twice with 150 mM NaCl. Results are expressed as a percentage of forskolin-stimulated cAMP accumulation for each cell line and treatment in the absence of carbachol. These data represent triplicate determinations in one experiment; one additional experiment gave similar results.

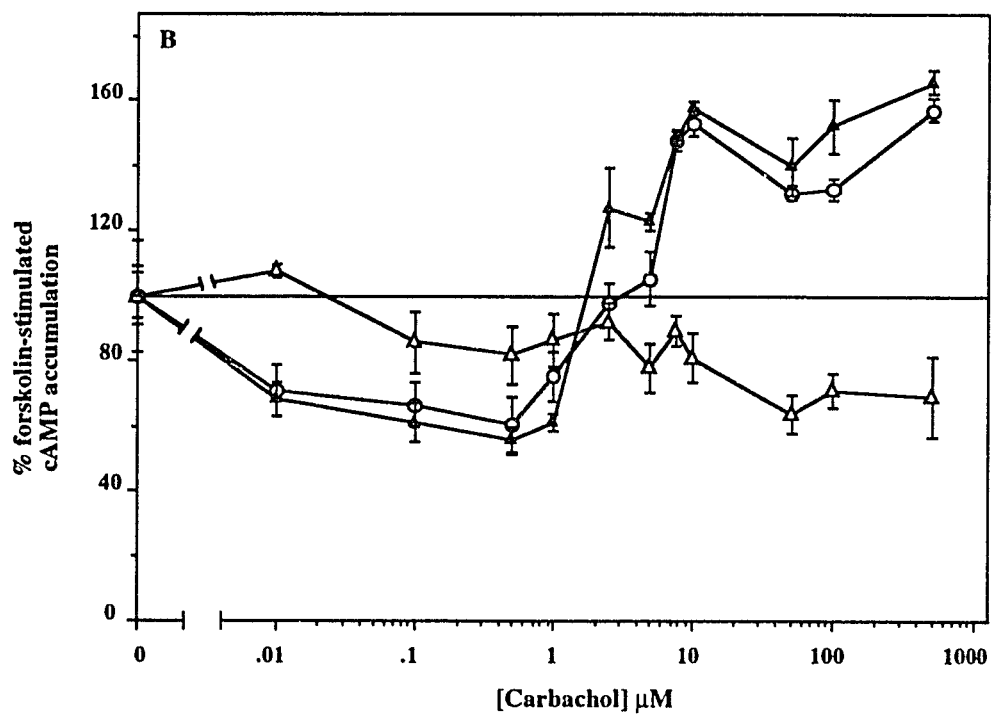
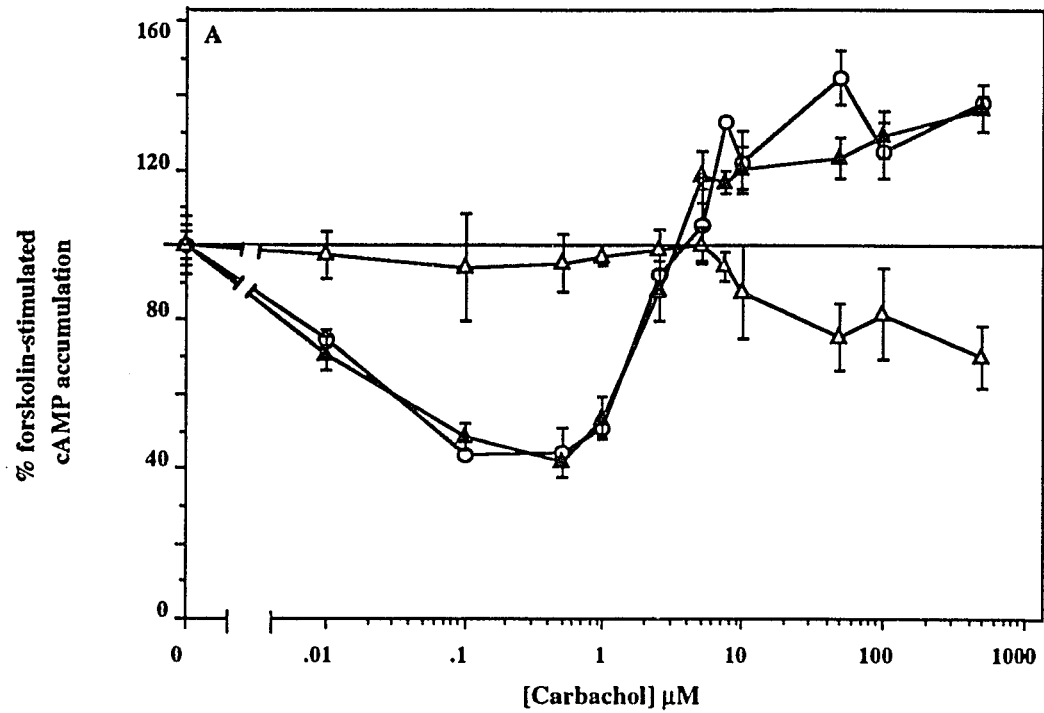


FIGURE 15. Effect of muscarinic agonist on adenylyl cyclase activity in membranes isolated from HEK-293 cells expressing type I adenylyl cyclase and m4 muscarinic receptors. Membranes isolated from cell lines expressing varying levels of m4 muscarinic receptor and type I adenylyl cyclase (P1-6 ▲; M1-37 ●, ○; M1-36 ■, □) were assayed for adenylyl cyclase activity in the presence of 10.0 μM GTP, 5.0 μM forskolin and increasing concentrations of carbachol. To ensure the effects of carbachol were mediated via muscarinic activation, membranes were also assayed at the highest carbachol concentration (1.0 mM) in the presence of 1.0 μM atropine (○, □). Results are expressed as a percentage of forskolin-stimulated adenylyl cyclase activity for each cell line and treatment in the absence of carbachol. Specific activities (pmol cAMP formed/min/mg) of forskolin-stimulated adenylyl cyclase activity in isolated membranes were P1-6: 149.82 ± 20.67, M1-37: 148.53 ± 6.49, M1-36: 134.40 ± 18.68. Membrane isolation and adenylyl cyclase assay are described under "Experimental Procedures." These data represent triplicate determinations in one experiment; two additional experiments gave similar results.

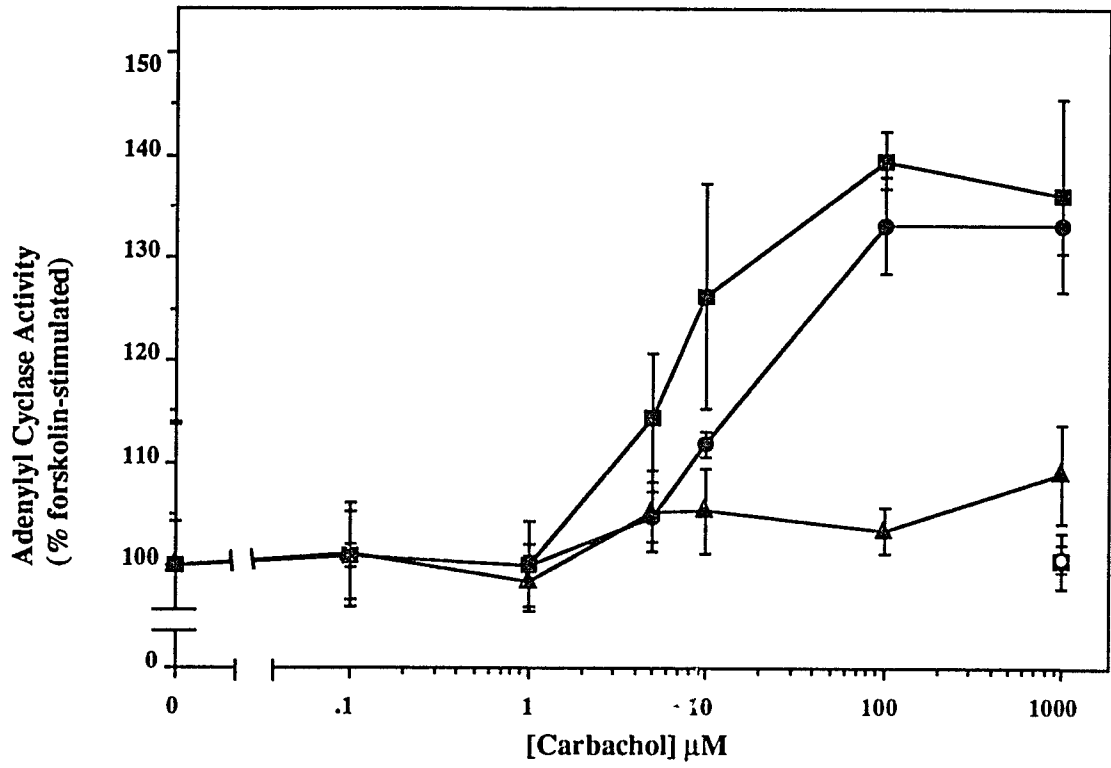


FIGURE 16. Effect of GDP-beta-S on carbachol stimulated adenylyl cyclase activity in membranes expressing type I adenylyl cyclase and m4 receptors. Membranes isolated from cell line M1-36 were assayed for adenylyl cyclase activity in the presence of 10.0 μ M GTP, 5.0 μ M forskolin and increasing concentrations of carbachol either in the presence (O) or absence (\square) of 50.0 μ M GDP-beta-S. To ensure the effects of carbachol were mediated via muscarinic activation, membranes were also assayed at the highest carbachol concentration (0.5 mM) in the presence of 1.0 μ M atropine. Membrane preparation and adenylyl cyclase assay are described under "Experimental Procedures." These data represent triplicate determinations in one experiment; one additional experiments gave similar results.

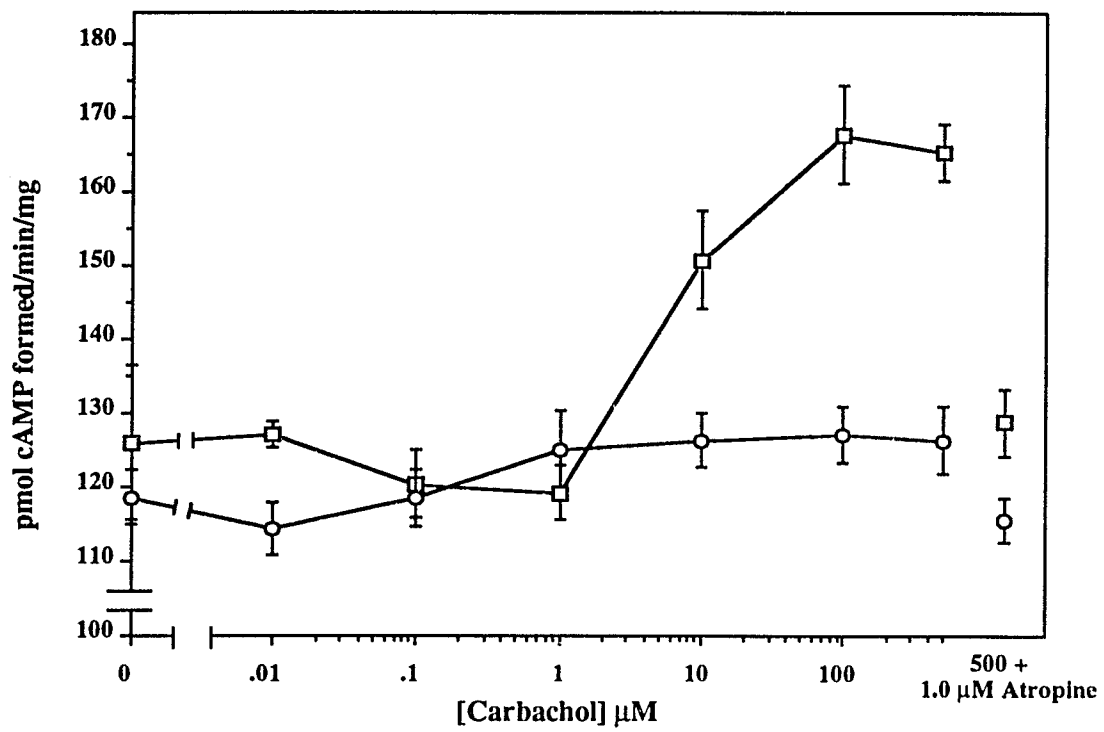


FIGURE 17. Carbachol-mediated stimulation of cyclic AMP accumulation in cells treated with PTX. Monolayers of cells expressing m4 receptors and either A) type I adenylyl cyclase (M1-37 (□, ■), M1-36 (○, ●, ▲) or B) type III adenylyl cyclase (M3-14 (○, ●), M3-32 (□, ■)) were incubated for 16-20 hours in the presence (■, ●, ▲) or absence (□, ○) of 100 ng/ml PTX in DMEM containing [³H] adenine. Immediately prior to carbachol exposure, PTX-containing media was removed, cells were incubated for 30 min. in the presence (▲) or absence (□, ■, ○, ●) of 100.0 μM BAPTA/AM in non-supplemented DMEM and washed twice with 150 mM NaCl. Cells were exposed to increasing concentrations of carbachol for 30 min. in incubation buffer containing 5.0 μM Forskolin and 1.0 mM IBMX and relative cAMP accumulations were determined as described in Experimental Procedures. Results are expressed as fold-stimulation of forskolin-stimulated cAMP accumulation for each cell line and treatment in the absence of carbachol. These data represent triplicate determinations in one experiment; one additional experiment gave similar results.

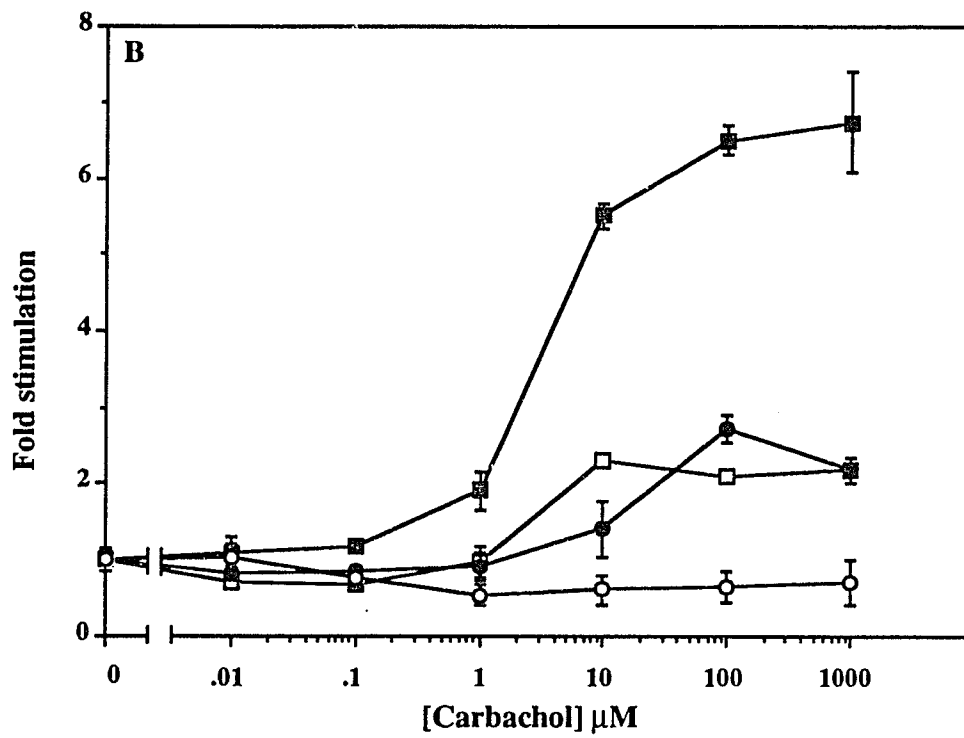
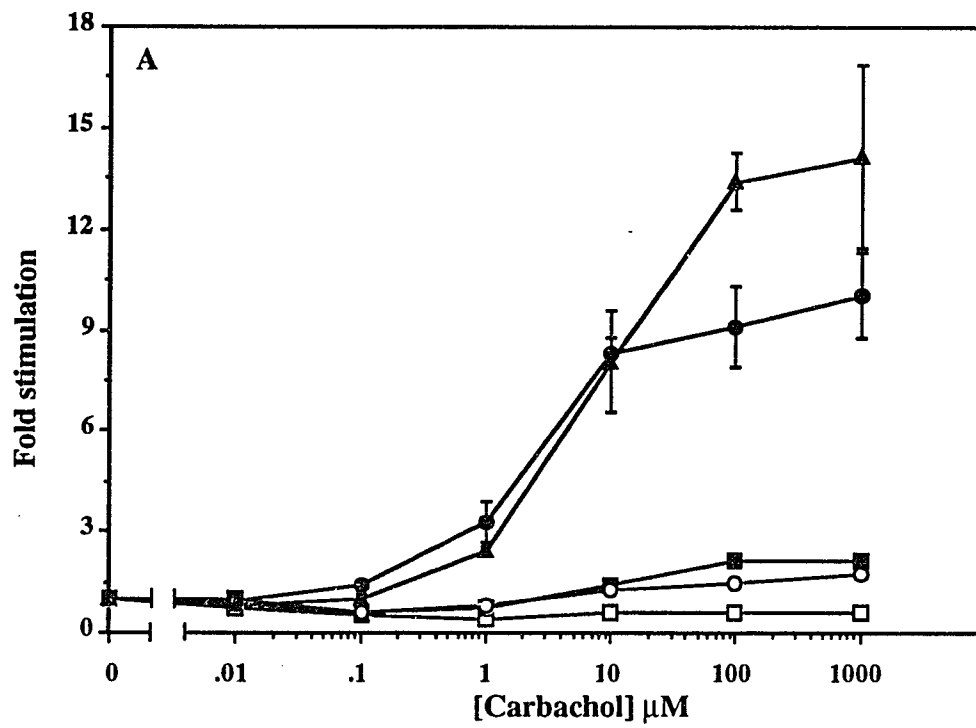
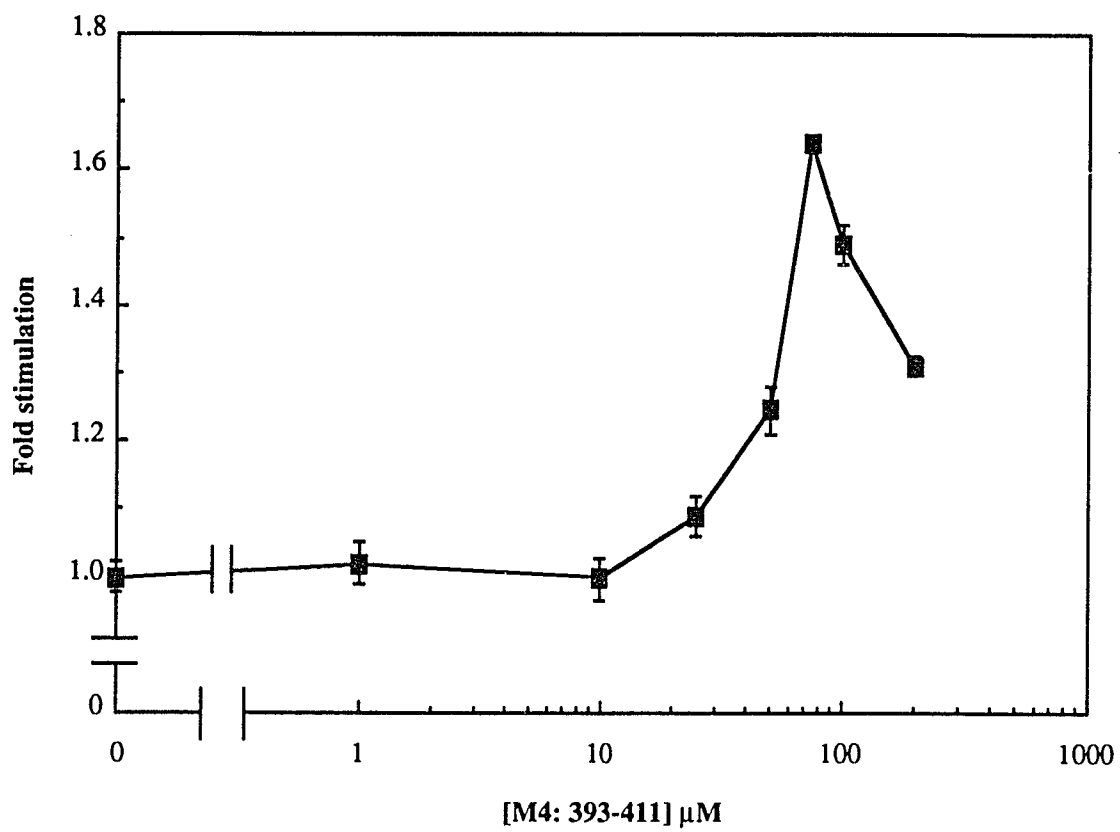


FIGURE 18. Effect of G protein activating peptide m4:393-411 on adenylyl cyclase activity in PTX-treated membranes expressing type III adenylyl cyclase. Monolayers of cells expressing type III adenylyl cyclase and no exogenous m4 receptor (P3-2) were incubated for 16-20 hours in the presence of 100 ng/ml PTX. Membranes isolated from these cells were assayed for adenylyl cyclase activity in the presence of 10.0 μ M GTP and increasing concentrations of m4:393-411. Membrane preparation and adenylyl cyclase assay are described under "Experimental Procedures." These data represent triplicate determinations in one experiment; one additional experiments gave similar results.



LITERATURE CITED

- Andersen, Ø. and Døving, K. B. (1991) Gonadotropin releasing hormone (GnRH) - a novel olfactory stimulant in fish. *NeuroReport* **2**: 458-460.
- Anholt, R. R. H. and Rivers, A. M. (1990) Olfactory transduction: cross-talk between second messenger systems. *Biochemistry* **29**: 4049-4054.
- Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J. and Capon, D. J. (1989a) Functional diversity of muscarinic receptor subtypes in cellular signal transduction and growth. *Trends Pharmacol. Sci.* **10**(suppl.): 16-22.
- Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J. and Capon, D. J. (1989b) Functionally distinct G proteins selectively couple different receptors to PI hydrolysis in the same cell. *Cell* **56**: 487-493.
- Bakalyar, H. A. and Reed, R. R. (1990) Identification of a specialized adenylyl cyclase that may mediate odorant detection. *Science* **250**: 1403-1406.
- Baumgold, J. (1992) Muscarinic receptor-mediated stimulation of adenylyl cyclase. *TIPS* **13**: 339-340.
- Baumgold, J., Paek, R. and Fiskum, G. (1992) Calcium independence of phosphoinositide hydrolysis induced increase in cyclic AMP accumulation in SK-N-SH human neuroblastoma cells. *J. Neurochem.* **58**: 1754-1759.
- Berman, C. H. and Quinn, T. P. (1991) Behavioural thermoregulation and homing by spring chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), in the Yakima River. *J. Fish Biol.* **39**: 301-312.
- Black, G. A. and Dempson, J. B. (1986) A test of the hypothesis of pheromone attraction in salmonid migration. *Env. Biol. Fish.* **15**: 229-235.

Bodznick, D. (1978) Water source preferences and lakeward migration of sockeye salmon fry (*Oncorhynchus nerka*). *J. Comp. Physiol.* **127**: 139-146.

Boekhoff, I., Tareilus, E., Strotmann, J. and Breer, H. (1990) Rapid activation of alternative second messenger pathways in olfactory cilia from rats by different odorants. *EMBO J.* **9**: 2453-2458.

Bonner, T. I., Young, A. C., Brann, M. R. and Buckley, N. J. (1988) Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1**: 403-410.

Bourne, H. R. and Nicoll, R. (1993) Molecular machines integrate coincident synaptic signals. *Neuron* **10** (suppl.): 65-75.

Brand, J. G. and Bruch, R. C. (1992) Molecular mechanisms of chemosensory transduction: gustation and olfaction. In *Fish Chemoreception* (Edited by Hara, T. J.), pp. 126-149. Chapman and Hall, London.

Brannon, E. L. (1972) Mechanisms controlling migration of sockeye salmon fry. *Int. Pac. Salmon Fish. Comm. Bull.* **21**: 86 p.

Brannon, E. L. (1982) Orientation mechanisms of homing salmonids, In *Salmon and trout migratory behavior symposium*, Brannon, E. L. and Salo, E. O. (eds.) School of Fisheries, University of Washington, Seattle, pp. 219-227.

Brannon, E. L. and Quinn, T. P. (1990) Field test of the pheromone hypothesis for homing by Pacific salmon. *J. Chem. Ecol.* **16**: 603-609.

Brannon, E. L., Feldman, C. and Donaldson, L. R. (1982) University of Washington zero-age coho salmon smolt production. *Aquaculture* **28**:195-200.

Breer, H., Boekhoff, I. and Tareilus, E. (1990) Rapid kinetics of second messenger formation in olfactory transduction. *Nature* **345**: 65-68.

Breer, H., Klemm, T. and Boekhoff, I. (1992) Nitric oxide mediated formation of cyclic GMP in the olfactory system. *NeuroReport* **3**: 1030-1032.

Breer, H. and Shepherd, G. M. (1993) Implications of the NO/cGMP system for olfaction. *TINS* **16**: 5-9.

Brostrom, C. O., Huang, Y. C., Breckenridge, B. M. and Wolfe, D. J. (1975) Identification of a calcium-binding protein as a calcium-dependent regulator of brain adenylate cyclase. *Proc. Natl. Acad. Sci.* **72**: 64-68.

Brown, H. J. and Rietow, M. (1981) Muscarinic-dopaminergic synergism on retinal cyclic AMP formation. *Brain Res.* **215**: 388-392.

Brown, S. B. and Hara, T. J. (1981) Accumulation of chemostimulatory amino acids by a sedimentable fraction isolated from olfactory rosettes of rainbow trout (*Salmo gairdneri*). *Biochim. Biophys. Acta* **675**: 149-162.

Bruch, R. C. and Teeter, J. H. (1989) Second messenger signalling mechanisms in olfaction. In *Chemical Senses I: Receptor Events and Transduction in Taste and Olfaction*, Brand, J. G., Teeter, J. H., Kare, M. R. and Cagan, R. H.(eds.), Marcel Dekker, New York, pp. 283-298.

Bruch, R. C. and Teeter, J. H. (1990) Cyclic AMP links amino acid chemoreceptors to ion channels in olfactory cilia. *Chem. Senses* **15**: 419-430.

Bruch, R. C., Rulli, R. D. and Boyle, A. G. (1987) Olfactory L-amino acid receptor specificity and stimulation of potential second messengers. *Chem. Senses* **12**: 642-643.

Buck, L. and Axel, R. (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* **65**: 175-187.

Chen, C. and Okayama, H. (1987). High-efficiency transfection of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**: 2745-2752.

- Chen, Z., Pace, U., Heldman, J., Shapira, A. and Lancet, D. (1986) Isolated frog olfactory cilia: a preparation of dendritic membranes from chemosensory neurons. *J. Neurosci.* **6**: 2146-2154.
- Choi, E. J., Xia, Z. and Storm, D. R. (1992a) Stimulation of the type III olfactory adenylyl cyclase by calcium and calmodulin. *Biochemistry* **31**: 6492-6498.
- Choi, E. J., Wong, S. T., Hinds, T.J. and Storm, D. R. (1992b). Calcium and muscarinic agonist stimulation of type I adenylyl cyclase in whole cells. *J. Biol. Chem.* **267**: 12440-12442.
- Choi, E. J., Xia, Z., Villacres, E. C. and Storm, D. R. (1993a) The regulatory diversity of the mammalian adenylyl cyclases. *Current Opinion in Cell Biol.* **5**: 269-273.
- Choi, E. J., Wong, S. T., Dittman, A. H. and Storm, D. R. (1993b) Phorbol ester stimulation of the type I and type III adenylyl cyclases in whole cells. *Biochemistry* **32**: 1891-1894.
- Clarke, W. C. and Blackburn, J. (1977) A seawater challenge test to measure smolting in juvenile salmon. *Dept. Envir., Fish. Mar. Serv. Res. Div. Dir., Tech. Rep.* **705**, 11 pp.
- Cooper, J. C. and Hasler, A. D. (1973) An electrophysiological approach to salmon homing. *Fish. Res. Bd. Can. Tech. Rep.* **415**: 44 p.
- Cooper, J. C., Scholz, A. T., Horrall, R. M., Hasler, A. D. and Madison, D. M. (1976) Experimental confirmation of the olfactory hypothesis with artificially imprinted homing coho salmon (*Oncorhynchus kisutch*). *J. Fish. Res. Bd. Can.* **33**: 703-710.
- Courtenay, S. C. (1989) *Learning and memory of chemosensory stimuli by underyearling coho salmon, Oncorhynchus kisutch Walbaum*. Ph.D. thesis, University of British Columbia, Vancouver, B.C., 231 pp.

Dhallan, R. S., Yau, K.-W., Schrader, K. A. and Reed, R. R. (1990) Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. *Nature* **347**: 184-187.

Dickhoff, W. W. and Sullivan, C. V. (1987) Involvement of the thyroid gland in smoltification, with special reference to metabolic and developmental processes. *Am. Fish. Soc. Symp.* **1**: 197-210.

Dickhoff, W. W., Folmar, L. C. and Gorbman, A. (1978) Changes in plasma thyroxine during smoltification of coho salmon, *Oncorhynchus kisutch*. *Gen. Comp. Endocrinol.* **36**: 229-232.

Dickhoff, W. W., Darling, D. S. and Gorbman, A. (1982) Thyroid function during smoltification of salmonid fish. *Gunma Symposia on Endocrinology* **19**: 45-61.

Donaldson L.R. and Allen, G. H. (1957) Return of silver salmon, *Oncorhynchus kisutch* (Walbaum) to point of release. *Trans. Am. Fish. Soc.* **87**: 13-22.

Dulka, J. G., Stacey, N. E., Sorensen, P. W. and Van Der Kraak, G. J. (1987) Sex steroid pheromone synchronizes male-female spawning readiness in the goldfish. *Nature* **325**: 251-253.

Duzic, E. and Lanier, S. M. (1992) Factors determining the specificity of signal transduction by guanine nucleotide-binding protein-coupled receptors III Coupling of α_2 -adrenergic receptor subtypes in a cell type-specific manner. *J. Biol. Chem.* **267**: 24045-24052.

Dye, H. M., Sumpter, J. P., Fagerlund, U. H. M. and Donaldson, E. M. (1986) Changes in reproductive parameters during the spawning migration of pink salmon, *Oncorhynchus gorbuscha* (Walbaum). *J. Fish Biol.* **29**: 167-176.

Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R. and Liggett, S. B. (1992) Simultaneous coupling of α_2 -adrenergic receptors to two G-proteins with opposing effects. *J. Biol. Chem.* **267**: 15795-15801.

Emanuel, M. E. and Dodson, J. J. (1979) Modification of the rheotropic behavior of male rainbow trout (*Salmo gairdneri*) by ovarian fluid. *J. Fish. Res. Board Can.* **36**: 63-68.

Federman, A. D., Conklin, B. R., Schrader, K. A., Reed, R. R. and Bourne, H. R. (1992) Hormonal stimulation of adenylyl cyclase through Gi-protein $\beta\gamma$ subunits. *Nature* **356**: 159-161.

Felder, C. C., Kanterman, R. Y., Ma, A. L. and Axelrod, J. (1989) A transfected m1 muscarinic acetylcholine receptor stimulates adenylate cyclase via phosphatidylinositol hydrolysis. *J. Biol. Chem.* **264**: 20356-20362.

Folmar, L. C. and Dickhoff, W. W. (1980) The parr-smolt transformation (smoltification) and seawater adaptation in salmonids; A review of selected literature. *Aquaculture* **21**: 1-37.

Folmar, L. C. and Dickhoff, W. W. (1981) Evaluation of some physiological parameters as predictive indices of smoltification. *Aquaculture* **23**: 309-324.

Foote, C. J. and Larkin P.A. (1988) The role of male choice in the assortative mating of anadromous and non-anadromous sockeye salmon, *Oncorhynchus nerka*. *Behaviour* **106**: 43-62.

Fraser, C.M, Arakawa, S., McCombie, W. R. and Venter, J. C. (1989) Cloning, sequence analysis, and permanent expression of a human α_2 -adrenergic receptor in chinese hamster ovary cells. *J. Biol. Chem.* **264**: 11754-11761.

Gebhards, S. V. (1960) Biological notes on precocious male chinook salmon parr in the Salmon River drainage, Idaho. *Prog. Fish-Cult.* **22**: 121-123.

Gilman, A. (1970) A protein binding assay for adenosine 3': 5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. USA* **67**: 305-312.

Glatt, C. E. and Snyder, S. H. (1993) Cloning and expression of an adenylyl cyclase localized to the corpus striatum. *Nature* **361**: 536-538.

Goetz, F. W., Fostier, A. Y., Breton, B. and Jalabert, B. (1987) Hormonal changes during meiotic maturation and ovulation in the brook trout (*Salvelinus fontinalis*). *Fish Physiol. Biochem.* **3**: 203-211.

Goulding, E. H., Ngai, J., Kramer, R. H., Colicos, S., Axel, R., Siegelbaum, S. A. and Chess, A. (1992) Molecular cloning and single-channel properties of the cyclic nucleotide-gated channel from catfish olfactory neurons. *Neuron* **8**: 45-58.

Grau E. G., Dickhoff, W. W., Nishioka, R. S., Bern, H. A. and Folmar, L. C. (1981) Lunar phasing of the thyroxine surge preparatory to seawater migration of salmonid fish. *Science* **211**: 607-609.

Grau, E. G., Specker, J. L., Nishioka, R. S. and Bern, H. A. (1982) Factors determining the occurrence of the surge in thyroid activity in salmon during smoltification. *Aquaculture* **28**: 49-57.

Grau, E. G., Fast, A., Nishioka, R. S. and Bern, H. A. (1985) The effects of transfer to novel conditions on blood thyroxine in coho salmon. *Aquaculture* **45**: 377.

Groot, C. and Margolis, L. (1991) *Pacific salmon life histories*. UBC press, University of British Columbia, Vancouver, BC. 564 p.

Groot, C., Quinn, T. P. and Hara, T. J. 1986. Responses of migrating adult sockeye salmon (*Oncorhynchus nerka*) to population-specific odors. *Can. J. Zool.* **64**: 926-932.

Gross, M. R. (1985) Disruptive selection for alternative life history in salmon. *Nature* **313**: 47-48.

Halvorsen, M. and Stabell, O. B. (1990) Homing behaviour of displaced stream-dwelling brown trout. *Anim. Behav.* **39**: 1089-1097.

- Hansen, L. P. and Jonsson, B. (1991) The effect of timing of Atlantic salmon smolt and postsmolt release on the distribution of adult return. *Aquaculture* **98**: 61-67.
- Hanson, A. J. and Smith, H. D. (1967) Mate selection in a population of sockeye salmon (*Oncorhynchus nerka*) of mixed age-groups. *J. Fish. Res. Board Can.* **24**: 1955-1977.
- Hara, T. J. (1972) Electrical responses of the olfactory bulb of Pacific salmon *Oncorhynchus nerka* and *Oncorhynchus kisutch*. *J. Fish. Res. Bd. Can.* **29**: 1351-1355.
- Hara, T. J. (1975) Olfaction in fish. *Prog. Neurobiol.* **5**: 271-335.
- Hara, T. J. (1982) Structure-activity relationships of amino acids as olfactory stimuli. In *Chemoreception in Fishes*, Hara, T. J.(ed.), Elsevier, Amsterdam, pp.135-158.
- Hara, T. J. (1992) Mechanisms of olfaction. In *Fish Chemoreception*, Hara, T. J.(ed.) Chapman and Hall, London, pp. 150-170.
- Hara, T. J. and Zielinski, B. (1989) Structural and functional development of the olfactory organ in teleosts. *Trans. Am. Fish. Soc.* **118**: 183-194.
- Harden Jones, F. R. (1968) *Fish Migration*, Arnold, London. 325 p.
- Hartman, G. F. (1969) Reproductive biology of the Gerrard stock of rainbow trout. In *Symp. on Salmon and Trout in Streams*, T. G. Northcote (ed.) H. R. MacMillan Lectures in Fisheries. University of British Columbia, Vancouver. pp. 39-51.
- Hasler, A. D. and Wisby, W. J. (1951) Discrimination of stream odors by fishes and relation to parent stream behavior. *Am. Naturalist* **85**: 223-238.
- Hasler A.D. and Scholz, A. T. (1983) *Olfactory imprinting and homing in salmon*. Springer-Verlag, Berlin. 134 p.
- Hassid, A. (1986) Atriopeptin II decreases cytosolic free Ca²⁺ in cultured vascular smooth muscle cells. *Am. J. Physiol.* **251**: C681-686.

Hille, B. (1992) G protein-coupled mechanisms and nervous signaling. *Neuron* **9**: 187-195.

Hoar, W. S. (1976) Smolt transformation: evolution, behavior, and physiology. *J. Fish. Res. Bd Can.* **33**: 1234-1252.

Hochachka, P. W. and Somero, G. N. (1971) Biochemical adaptation to the environment. In *Fish Physiology, Vol VI, Environmental Relations and Behavior*, Hoar, W. S. and Randall, D. J.(eds.) Academic Press, New York, pp. 100-156.

Hoffnagle, T. L. and Fivizzani, A.J. (1990) Stimulation of plasma thyroxine levels by novel water chemistry during smoltification in chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat. Sci.* **47**: 1513-1517.

Honda, H. (1980) Female sex pheromone of rainbow trout, *Salmo gairdneri*, involved in courtship behaviour. *Bull. Jap. Soc. Sci. Fish.* **46**: 1109-1112.

Honda, H. (1982) On the female sex pheromones and courtship behaviour in the salmonids, *Oncorhynchus masou* and *O. rhodurus*. *Bull. Jap. Soc. Sci. Fish.* **48**: 47-49.

Huque, T. and Bruch, R. C. (1986) Odorant- and guanine nucleotide-stimulated phosphoinositide turnover in olfactory cilia. *Biochem. Biophys. Res. Commun.* **137**: 36-42.

Hutchings, J. A. and Myers, R. A. (1988) Mating success of alternate maturation phenotypes in male Atlantic salmon, *Salmo salar*. *Oecologia* **75**: 169-174.

Ikezu, T., Okamoto, T., Ogata, E. and Nishimoto, I. (1992) Amino acids 356-372 constitute a Gi-activator sequence of the α_2 -adrenergic receptor and have a Phe substitute in the G protein-activator sequence motif. *FEBS Lett.* **311**: 29-32.

- Iwamoto, R. N. (1982) *Strain-photoperiod-temperature interactions in coho salmon: freshwater growth, smoltification and seawater adaptation*. Ph.D. thesis, University of Washington, Seattle WA.
- Jansson, C. C., Kukkonen, J. and Akerman, K. E. O. (1991) Muscarinic receptor-linked elevation of cAMP in SH-SY5Y neuroblastoma cells is mediated by Ca²⁺ and protein kinase C. *Biochim. Biophys. Acta* **1095**: 255-260.
- Jensen, A. L. and Duncan, R. N. (1971) Homing of transplanted coho salmon. *Prog. Fish-Cult.* **33**: 216-218.
- Johnsen, P. B. and Hasler, A. D. (1980) The use of chemical cues in the upstream migration of coho salmon, *Oncorhynchus kisutch* Walbaum. *J. Fish Biol.* **17**: 67-73.
- Johnson, S. L., Solazzi, M. F. and Nickelson, T. E. (1990) Effects on survival and homing of trucking hatchery yearling coho salmon to release sites. *N. Amer. J. Fish. Manag.* **10**: 427-433.
- Jones, S. V. P., Heilman, C. J. and Brann, M. R. (1991) Functional responses of cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharm.* **40**: 242-247.
- Jones, S. V. P., Levey, A. I., Weiner, D. M., Ellis, J., Novotny, E., Yu, S-H., Dorje, F., Wess, J. and Brann, M. R. (1992) Muscarinic acetylcholine receptors, in *Molecular Biology of G-protein-coupled receptors*, M. R. Brann (ed.) Birkhauser, Boston, 170-197.
- Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W.-J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R. and Gilman, A. G. (1989) Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. *Science* **244**: 1558-1564.
- Lin, R. J., Rivas, R. J., Grau, E. G., Nishioka, R. S. and Bern, H. A. (1985) Changes in plasma thyroxine following transfer of young coho salmon (*Oncorhynchus kisutch*) from fresh water to fresh water. *Aquaculture* **45**: 381-382.

- Lin, R. J., Cross, T. F., Mills, C. P. R., Nishioka, R. S., Grau, E. G. and Bern, H. A. (1988) Changes in plasma thyroxine levels during smoltification in hatchery-reared one-year and two-year Atlantic salmon (*Salmo salar*). *Aquaculture* **74**: 369-378.
- Lichtshtein, D., Boone, G. and Blume, A. (1979) Muscarinic receptor regulation of NG108-15 adenylate cyclase: requirement for Na⁺ and GTP. *J. Cyclic Nucleotide Res.* **5**: 367-375.
- Liley, N. R. (1982) Chemical communication in fish. *Can. J. Fish. Aquat. Sci.* **39**:22-35.
- Liley, N. R., Fostier, A., Breton, B. and Tan, E. S. P. (1986a) Endocrine changes associated with spawning behavior and social stimuli in a wild population of rainbow trout (*Salmo gairdneri*) II. Females. *Gen. Comp. Endocrinol.* **62**: 157-167.
- Liley, N. R., Breton, B., Fostier, A. and Tan, E. S. P. (1986b) Endocrine changes associated with spawning behavior and social stimuli in a wild population of rainbow trout (*Salmo gairdneri*) I. Males. *Gen. Comp. Endocrinol.* **62**: 145-156.
- Liley, N. R., Olsen, H. K. and Foote, C. J. (1991) Reproductive pheromones in rainbow trout, *Oncorhynchus mykiss*, and kokanee salmon, *O. nerka*. In *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish.*, Scott, A. P., Sumpter, J. P., Kime, D. E., and Rolfe, M. S. (eds.) FishSymp 91, Sheffield. pp. 188-190.
- Lister, D. B., Hickey, D.G. and Wallace, I. (1981) *Review of the effects of enhancement strategies on the homing, straying and survival of Pacific salmonids*. MS Rept. prepared for the Can. Dept. Fish. and Oceans.
- Lo, Y. H., Bradley, T. M. and Rhoads, D. E. (1991) L-alanine binding sites and Na⁺,K⁺-ATPase in cilia and other membrane fractions from olfactory rosettes of Atlantic salmon. *Comp. Biochem. Physiol.* **98B**: 121-126.
- Lo, Y. H., Bradley, T. M. and Rhoads, D. E. (1993) Stimulation of Ca²⁺-regulated olfactory phospholipase C by amino acids. *Biochemistry* **32**: 123-58-12362.

- Maekawa, K and Onozato, H. (1986) Reproductive tactics and fertilization success of mature male Miyabe charr, *Salvelinus malma miyabei*. *Énv. Biol. Fish.* **15**: 119-129.
- McCormick, S. D. and Saunders, R. L. (1990) Influence of ration level and salinity on circulating thyroid hormones in juvenile Atlantic salmon (*Salmo salar*). *Gen. Comp. Endocrinol.* **78**: 224-230.
- Moore, A. (1991) Behavioural and physiological responses of precocious male Atlantic salmon (*Salmo salar* L.) parr to testosterone. In *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish*, (A. P. Scott, J. P. Sumpter, D. E. Kime, and M. S. Rolfe (eds.) FishSymp 91, Sheffield. pp. 194-196.
- Moore, A. and Scott, A. P. (1991) Testosterone is a potent odorant in precocious male Atlantic salmon (*Salmo salar* L.) parr. *Phil. Trans. R. Soc. Lond. B.* **332**: 241-244.
- Moore, A. and Scott, A. P. (1992) $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one 20-sulphate is a potent odorant in precocious male Atlantic salmon parr (*Salmo salar*) which have been pre-exposed to the urine of ovulated females. *Proceedings of the Royal Society of London, Series B* **249**: 205-209.
- Morin P.-P., Dodson, J. J. and Dore, F. Y. (1989a) Cardiac responses to a natural odorant as evidence of a sensitive period for olfactory imprinting in young Atlantic salmon, *Salmo salar*. *Can. J. Fish. Aquat. Sci.* **46**: 122-130.
- Morin P.-P., Dodson, J. J. and Dore, F. Y. (1989b) Thyroid activity concomitant with olfactory learning and heart rate changes in Atlantic salmon, *Salmo salar*, during smoltification. *Can. J. Fish. Aquat. Sci.* **46**: 131-136.
- Murray, C. P. and Rosenau, M. L. (1989) Rearing of juvenile chinook salmon (*Oncorhynchus tshawytscha*) in non-natal tributaries of the lower Fraser River, British Columbia. *Trans. Am. Fish. Soc.* **118**: 284-289.

Nakamura, T. and Gold, G. H. (1987) A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature* **325**: 442-444.

Nathanson, N. M. (1987) Molecular properties of the muscarinic acetylcholine receptor. *Annu. Rev. Neurosci.* **10**: 195-236.

Nathanson, N. M., Goldman, P.S., Habecker, B. A., Migeon, J. C., Morton, M. E., Tietje, K. M. and van Koppen, C. J. (1992). Analysis of muscarinic acetylcholine receptor expression and function. *Methods in Neurosciences* **9**: 116-134.

Nesbitt, J. A., Anderson, W. B., Miller, Z., Russell, T. R., Gospodarowicz, D. and Pastan, I. (1976) Guanylate cyclase and cyclic guanosine 3':5'-monophosphate phosphodiesterase activities and cyclic guanosine 3':5'-monophosphate levels in normal and transformed fibroblasts in culture. *J. Biol. Chem.* **251**: 2344-2352.

Newcombe, C. and Hartman, G. (1973) Some chemical signals in the spawning behavior of rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.* **30**: 995-997.

Nevitt, G. A. (1990) *Peripheral mechanisms of olfaction in two teleost fishes*. Ph.D. thesis, University of Washington, WA.

Nevitt, G. A. and Moody, W. J. (1992) An electrophysiological characterization of ciliated olfactory receptor cells of the coho salmon *Oncorhynchus kisutch*. *J. Exp. Biol.* **166**: 1-17.

Nevitt, G. A., Dittman, A. H., Quinn, T. P. and Moody, W. J. (in press). Evidence for a peripheral olfactory memory in imprinted salmon. *Proc. Natl. Acad. Sci.*

Ngai, J., Dowling, M. M., Buck, L., Axel, R. and Chess, A. (1993) The family of genes encoding odorant receptors in the channel catfish. *Cell* **72**: 657-666.

Nishikawa, K., Hirashima, T., Suzuki, S. and Suzuki, M. (1979) Changes in circulating L-thyroxine and L-triiodothyronine of the masu salmon (*Oncorhynchus masou*)

accompanying the smoltification, measured by radioimmunoassay. *Endocrinol. Jap.* **26**: 731-735.

Nishioka, R. S., Young, G., Bern, H. A., Jochimsen, W. and Hiser, C. (1985) Attempts to intensify the thyroxin surge in coho and king salmon by chemical stimulation. *Aquaculture* **45**: 215-225.

Nishioka, R. S., Grau, E. G., Bern, H. A., Lin, R. J., Hubbell, P. M., Knutson, A. C., Hiser, C., Maria, D., Rightmier, M. and Jochimsen, W. (1989) Effect of lunar-phased releases on adult recovery of coho salmon from Trinity River and Iron Gate hatcheries in California. *Aquaculture* **82**: 355-365.

Nordeng, H. (1971) Is the local orientation of anadromous fishes determined by pheromones? *Nature* **233**: 411-413.

Nordeng, H. (1977) A pheromone hypothesis for homeward migration in anadromous salmonids. *Oikos* **28**: 155-159.

Okamoto, T., and Nishimoto, I. (1992) Detection of G protein-activator regions in M4 subtype muscarinic, cholinergic and α_2 -adrenergic receptors based upon characteristics in primary structure. *J. Biol. Chem.* **267**: 8342-8346.

Okamoto, T., Murayama, Y., Hayashi, Y., Inagaki, M., Ogata, E. and Nishimoto, I. (1991) Identification of a Gs activator region of the β_2 -adrenergic receptor that is autoregulated via protein kinase A-dependent phosphorylation. *Cell* **67**: 723-730.

Olianas, M. C., and Onali, P. (1990) Ca^{2+} -independent stimulation of adenylate cyclase activity by muscarinic receptors in rat olfactory bulb. *J. Neurochem.* **55**: 1083-1086.

Olianas, M. C., and Onali, P. (1991) Muscarinic stimulation of adenylate cyclase activity of rat olfactory bulb. II Characterization of the antagonist sensitivity and comparison with muscarinic inhibitions of the enzyme in striatum and heart. *J. Pharmacol. Exp. Ther.* **259**: 680-686.

- Olianas, M. C., and Onali, P. (1992) Properties of muscarinic-stimulated adenylate cyclase activity in rat olfactory bulb. *J. Neurochem.* **58**: 1723-1729.
- Olsen., K. H. (1986) Chemo-attraction between juveniles of two sympatric stocks of Arctic char, *Salvelinus alpinus* (L.), and their gene frequency of serum esterases. *J. Fish Biol.* **28**: 221-231.
- Olsen, K. H. (1992) Kin recognition in fish mediated by chemical cues. In *Fish Chemoreception*, T. J. Hara (ed.) Chapman and Hall, London. pp. 229-248.
- Olsen, K. H. and Liley, N. R. (1993) The significance of olfaction and social cues in milt availability, sexual hormone status, and spawning behavior of male rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* **89**:107-118.
- Pace, U., Hanski, E., Salomon, Y and Lancet, D. (1985) Odorant-sensitive adenylate cyclase may mediate olfactory reception. *Nature* **316**: 255-258.
- Pascual, M.A.(1993) *The estimation of salmon population parameters from coded wire tag data*. PhD Thesis University of Washington, Seattle, WA.
- Peralta, E. G., Ashkenazi, A., Winslow, J. W., Ramachandran, J. and Capon, D. J. (1988) Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. *Nature* **334**: 434-437.
- Pete, K. (1977) *Species-specific odor as a guiding mechanism for local orientation in homing chinook (Oncorhynchus tshawytscha) and coho (O. kisutch) salmon*. M.S. thesis, University of Washington, Seattle.
- Peterson, G. L. (1983) Determination of total protein. *Methods in Enzymol.* **91**: 95-119.
- Peterson, N. P. (1982) Immigration of juvenile coho salmon (*Oncorhynchus kisutch*) into riverine ponds. *Can. J. Fish. Aquat. Sci.* **39**: 1308-1310.

- Quinn, T. P. (1990) Current controversies in the study of salmon homing. *Ethol. Ecol. Evol.* **2**: 49-63.
- Quinn, T. P. and Busack, C. A. (1985) Chemosensory recognition of siblings in juvenile coho salmon (*Oncorhynchus kisutch*). *Anim. Behav.* **33**: 51-56.
- Quinn, T. P. and Hara, T. J. (1986) Sibling recognition and olfactory sensitivity in juvenile coho salmon (*Oncorhynchus kisutch*). *Can. J. Zool.* **64**: 921-925.
- Quinn, T. P. and Tolson, G. M. (1986) Evidence of chemically mediated population recognition in coho salmon (*Oncorhynchus kisutch*). *Can. J. Zool.* **64**: 84-87.
- Quinn, T. P. and Dittman, A. H. (1990) Pacific salmon migrations and homing: mechanisms and adaptive significance. *TREE* **5**: 174-177.
- Quinn, T. P. and Foote, C. J. (in press) The effects of body size and sexual dimorphism on the reproductive behaviour of sockeye salmon, *Oncorhynchus nerka*. *Animal Behaviour*.
- Quinn, T. P., Brannon, E. L. and Whitman, R. P. (1983) Pheromones and the water source preferences of adult coho salmon, *Oncorhynchus kisutch* Walbaum. *J. Fish Biol.* **22**: 677-684.
- Quinn, T. P., Brannon, E. L. and Dittman, A. H. (1989) Spatial aspects of imprinting and homing in coho salmon (*Oncorhynchus kisutch*). *Fish. Bull.* **87**: 769-74.
- Rehnberg, B. G. and Schreck, C. B. (1986) The olfactory L-serine receptor in coho salmon: biochemical specificity and behavioral response. *J. Comp. Physiol.* **159**: 61-67.
- Restrepo, D., Boekhoff, I. and Breer, H. (1993) Rapid kinetic measurements of second messenger formation in olfactory cilia from channel catfish. *Am. J. Physiol.* **264C**: 906-911.

Rhein, L. D. and Cagan, R. H. (1980) Biochemical studies of olfaction: isolation, characterization and odorant binding activity of cilia from rainbow trout olfactory rosettes. *Proc. Natl. Acad. Sci.* **77**: 4412-4416.

Rhein, L. D. and Cagan, R. H. (1983) Biochemical studies of olfaction: binding specificity of odorants to a cilia preparation from rainbow trout olfactory rosettes. *J. Neurochem.* **41**: 569-577.

Ross, E. M. (1989) Signal sorting and amplification through G protein-coupled receptors. *Neuron* **3**: 141-152.

Rouger, Y. and Liley, N. R. (1993) Effect of social environment on plasma hormones and availability of milt in spawning male rainbow trout (*Oncorhynchus mykiss* Walbaum) *Can. J. Zool.* **71**: 280-285.

Salomon, Y., Londos, C. and Rodbell, M. (1974) A highly sensitive adenylate cyclase assay. *Analyt. Biochem.* **58**: 541-548.

Scholz, A. T. (1980) *Hormonal regulation of smolt transformation and olfactory imprinting in coho salmon*. Ph.D. Thesis, University of Wisconsin. Madison, WI.

Scholz, A. T., Horrall, R. M., Cooper, J. C. and Hasler, A. D. (1976) Imprinting to chemical cues: the basis for home stream selection in salmon. *Science* **192**: 1247-1249.

Scholz, A. T., White, R. J., Muzi, M. and Smith, T. (1985) Uptake of radiolabelled triiodothyronine in the brain of steelhead trout (*Salmo gairdneri*) during parr-smolt transformation: implications for the mechanism of thyroid activation of olfactory imprinting. *Aquaculture* **45**: 199-214.

Scott, A. P., Sumpter, J. P. and Hardiman, P. A. (1983) Hormone changes during ovulation in the rainbow trout (*Salmo gairdneri* Richardson). *Gen. Comp. Endocrinol.* **49**: 128-134

- Scott, A. P. and Canario, A. V. M. (1992) $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one 20-sulphate: a major new metabolite of the teleost oocyte maturation-inducing steroid. *Gen. Comp. Endocrinol.* **85**:91-100.
- Scott, A. P., Liley, N. R. and Vermeirssen, E. L. M. (in press) Pheromonal effects of female urine and of synthetic $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one 20-sulphate on plasma sex steroid levels, gonadotrophin II levels and milt volumes in male rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Biol.*
- Selset, R. and Døving, K. B. (1980) Behaviour of mature anadromous char (*Salmo alpinus* L.) towards odorants produced by smolts of their own population. *Acta Physiol. Scand.* **108**: 113-122.
- Sklar, P. B., Anholt, R. R. H. and Snyder, S. H. (1986) The odorant-sensitive adenylate cyclase of olfactory receptor cells: differential stimulation by distinct classes of odorants. *J. Biol. Chem.* **261**: 15538-15543.
- Slatick, E., Gilbreath, L. G., Harmon, J. R., Bjornn, T. C., Ringe, R. R., Walch, K. A., Novotny, A. J. and Zaugg, W. S. (1988) *Imprinting hatchery reared salmon and steelhead trout for homing, 1978-1983*. Report prepared for Bonneville Power Administration, Project No. 78-1.
- Solazzi, M. F., Nickelson, T. E. and Johnson, S. L. (1991) Survival, contribution, and return of hatchery coho salmon (*Oncorhynchus kisutch*) released into freshwater, estuarine, and marine environments. *Can. J. Fish. Aquat. Sci.* **48**: 248-253.
- Sorensen, P. W. (1992a) Hormonally derived sex pheromones in goldfish: A mode for understanding the evolution of sex pheromone systems in fish. *Biol. Bull.* **183**: 173-177
- Sorensen, P. W. (1992b) Hormones, pheromones and chemoreception. In *Fish Chemoreception*, T. J. Hara (ed.) Chapman and Hall, London. pp. 199-228.
- Sorensen, P. W., Goetz, F. W., Scott, A. P. and Stacey, N. E. (1991) Recent studies indicate that goldfish use mixtures of unmodified hormones and hormonal metabolites as

sex pheromones. In *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish*, Scott, A. P., Sumpter, J. P., Kime, D. E. and Rolfe, M. S. (eds.) Sheffield: FishSymp 91. pp. 191-193.

Specker, J. L. and Schreck, C. B. (1984) Influence of ambient salts on plasma thyroxine concentration in juvenile coho salmon. *Trans. Am. Fish. Soc.* **113**: 90-94.

Specker, J. L., Brown, C. L., and Bern, H. A. (1992) Asynchrony of changes in tissue and plasma thyroid hormones during the parr-smolt transformation of coho salmon. *Gen. Comp. Endocrinol.* **88**: 397-405.

Stacey, N. E., Cradwell, J. R., Liley, N. R., Scott, A. P. and Sorensen, P. W. (1993) Hormones as sex pheromones in fish. *12th Int. Cong. Comp. Endocrin.*, Toronto, Canada.

Steilen, S., Klumpp, S. and Schultz, E. (1990) Guanylate cyclase in olfactory cilia from rat and pig. *Biochim. Biophys. Acta* **1054**: 69-72.

Stuart-Kregor, P. A. C., Sumpter, J. P. and Dodd, J. M. (1981) The involvement of gonadotrophin and sex steroids in the control of reproduction in the parr and adults of Atlantic salmon, *Salmo salar* L. *J. Fish Biol.* **18**: 59-72.

Sullivan, C. V. (1986) *Effects of the thyroid hormone triiodothyronine and the anti-thyroid drug propylthiouracil on thyroid system function, smoltification, and marine survival of coho salmon (Oncorhynchus kisutch)*. Ph.D. thesis, University of Washington, Seattle, WA.

Sullivan, C. V., Darling, D. S. and Dickhoff, W. W. (1987) Effects of triiodothyronine and propylthiouracil on thyroid function and smoltification of coho salmon (*Oncorhynchus kisutch*). *Fish Physiol. Biochem.* **4**: 121-135

Tang, W.-J. and Gilman, A. G. (1991) Type-specific regulation of adenylyl cyclase by G protein bg subunits. *Science* **254**: 1500-1503.

- Taussig, R. T., Quarmby, L. R. and Gilman, A. G. (1993) Regulation of purified type I and type II adenylyl cyclases by G protein beta/gamma subunits. *J. Biol. Chem.* **268**: 9-12.
- Tietje, K. M., Goldman, P. S. and Nathanson, N. M. (1990) Cloning and functional analysis of a gene encoding a novel muscarinic acetylcholine receptor expressed in chick heart and brain. *J. Biol. Chem.* **265**: 2828-2834.
- Ueda, H., Nagahama, Y., Tashiro, F. and Crim, L. (1983) Some endocrine aspects of precocious sexual maturation in amago salmon *Oncorhynchus rhodorus*. *Bull. Jap. Soc. Sci. Fish.* **49**: 587-596.
- Ueda, H., Hiroi, O., Hara, A., Yamauchi, K. and Nagahama, Y. (1984) Changes in serum concentrations of steroid hormones, thyroxine, and vitellogenin during spawning migration of the chum salmon, *Oncorhynchus keta*. *Gen. Comp. Endocrinol.* **53**: 203-211.
- Unwin, M. J. and Quinn, T. P. (1993) Homing and straying patterns of chinook salmon (*Oncorhynchus tshawytscha*) from a New Zealand hatchery: spatial distribution of strays and effects of release date. *Can. J. Fish. Aquat. Sci.* **50**: 1168-1175.
- Wall, S. J., Yasuda, R. P., Li, M., Ciesla, W. and Wolfe, B. B. (1992a) The ontogeny of m1-m5 muscarinic receptor subtypes in rat forebrain. *Dev. Brain Res.* **66**: 181-185.
- Wall, S. J., Yasuda, R. P., Li, M., Ciesla, W. and Wolfe, B. B. (1992b) Differential regulation of subtypes m1-m5 of muscarinic receptors in forebrain by chronic atropine administration. *J. Pharm. Exp. Therap.* **262**: 584-588.
- Wisby, W. J. and Hasler, A. D. (1954) Effect of olfactory occlusion on migrating silver salmon (*Oncorhynchus kisutch*). *J. Fish. Res. Bd. Can.* **11**: 472-478.
- Wong, Y. H., Federman, A., Pace, A. M., Zachary, I., Evans, T., Pouyssegur, J. Bourne, H. R. (1991) Mutant α subunits of G_{i2} inhibit cyclic AMP accumulation. *Nature* **351**: 63-65.

Wright, R. S. and Hunt, S. M. V. (1982) A radioimmunoassay for $17\alpha,20\beta$ -Dihydroxy-4-pregnene-3-one: Its use in measuring changes in serum levels at ovulation in atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), and rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol.* **47**: 475-482.

Xia, Z., Refsdal, C. D., Merchant, K. M., Dorsa, D. M. and Storm, D. R. (1991) Distribution of mRNA for the calmodulin-sensitive adenylate cyclase in rat brain: expression in areas associated with learning and memory. *Neuron* **6**: 431-443.

Yamauchi, K., Koide, K., Adachi, S. and Nagahama, Y. (1984) Changes in seawater adaptability and blood thyroxine concentrations during smoltification of the masu salmon, *Oncorhynchus masou*, and the amago salmon, *Oncorhynchus rhodrus*. *Aquaculture* **42**: 247-256.

Yamauchi, K., Kudo, H., Shimizu, M., Tsuneyoshi, Y. and Ueda, H. (1993) Biochemical and endocrinological aspects of olfactory imprinting in salmonids. Abstract 27, *International Conference and Workshop; Homing and Straying in salmon*. Roros, Norway.

Youngson, A. F. and Simpson, T. H. (1984) Changes in serum thyroxine levels during smolting in captive and wild Atlantic salmon, *Salmo salar* L. *J. Fish Biol.* **24**: 29-39.

Zar, J. H. (1984) *Biostatistical Analysis*. Prentice-Hall Inc., Englewood, N. J.

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Academic Training and Work Experience

Ph.D.	Special Individual PhD Fisheries/Pharmacology	1986-present	Dr. Thomas P. Quinn, Dr. Daniel R. Storm School of Fisheries, University of Washington, Seattle, WA
B.A.	Biochemistry	1978-1982	Dartmouth College, Hanover, NH
Research Technician		1982-1986	William A. Catterall, Daniel R. Storm, Department of Pharmacology, University of Washington, Seattle WA

Fellowships, Honors, Awards

1992	Lauren R. Donaldson Scholarship
1992	School of Fisheries Research Grant
1991	John N. Cobb Memorial Scholarship (Fisheries)
1991	School of Fisheries Research Award
1990,1988	School of Fisheries Travel Award
1989-present	NIH Environmental Pathology/Toxicology Training Grant
1989	John N. Cobb Memorial Scholarship (Fisheries)
1988	W. F. Thompson Memorial Scholarship (Fisheries)
1987	Melvin G. Anderson Memorial Scholarship (Fisheries)
1986	Claire L. and Evelyn S. Egtvedt Entering Scholar Award

Publications

Quinn, T. P., Brannon, E. L. and A. H. Dittman. 1989. Spatial Aspects of Imprinting and Homing in Coho Salmon, *Oncorhynchus kisutch*. Fish. Bull., 87: 769-774.

Quinn, T. P. and A. H. Dittman. 1990. Pacific Salmon Migrations and Homing: Mechanisms and Adaptive Significance. Trends in Evolution and Ecology. 5(6): 174-177.

Quinn, T. P. and A. H. Dittman. 1992. Homing in Fishes. in *Animal Homing*, F. Papi (ed.) Chapman Hall Publ., London. pp. 145-211.

Choi, E. J., Wong, S. T., Dittman, A. H. and D. R. Storm. 1993. Phorbol ester stimulation of the type I and type III adenylyl cyclases in whole cells. *Biochemistry*. 32: 1891-1894.

Dittman, A.H., Weber, J., Hinds, T. R., Choi, E-J, Migeon, J. C., Nathanson, N. M. and D.R. Storm. 1994 A novel mechanism for coupling m4 muscarinic acetylcholine receptors to calmodulin sensitive adenylyl cyclases: cross-over from G protein coupled inhibition to stimulation. *Biochemistry* 33: 943-951.

Dittman, A. H. and T. P. Quinn. (in press) Avoidance of a putative pheromone, 17a, 20b-dihydroxy- 4-pregnen-3-one, by precociously maturing male chinook salmon, *Oncorhynchus tshawytscha* . *Can. J. Zool.*

Duhe, R. J., Nielson, M., Dittman, A. H., Villeacres, E. C., Choi, E-J. and D. R. Storm. (in press) Oxidation of critical cysteine residues of type I adenylyl cyclases by nitric oxide reversibly inhibits stimulation by calcium and calmodulin. *J. Biol. Chem.*

Nevitt, G. A., Dittman, A. H., Quinn, T. P. and W. Moody. (in press) Evidence for a peripheral olfactory memory in imprinted salmon. *Proc. Natl. Acad.Sci.*

Dittman, A. H., Quinn, T. P., Dickhoff, W. W. and D. Larson. (submitted) Interactions between novel water, thyroxine, and olfactory imprinting in underyearling coho salmon *Oncorhynchus kisutch*.. *Aquaculture and Fish. Manage.*

Duhe, R. J., Dittman, A. H., Wu, Z and D. R. Storm. (invited review-submitted) Regulation of the mammalian adenylyl cyclases. *Treatise on Biomembranes*, Volume 4.

Quinn, T. P., Dittman, A. H., Peterson, P. and E. Volk. (submitted) Spatial distribution, survival and growth of sibling groups of juvenile coho salmon (*Oncorhynchus kisutch*) in an experimental stream channel. *Anim. Behav.*