

ASSESSMENT OF PRODUCTION OF CHUM SALMON FRY
FROM THE BIG BEEF CREEK SPAWNING CHANNEL

by

Gary J. Duker and Michael W. Colley

ANNUAL REPORT
Anadromous Fish Project

Project No. AFC-97
Contract No. 80-ABD-UW1B
Project Period: October 1, 1979 to September 30, 1980

Copies are available on request
for cost of duplication - \$2.80.

FRI-UW-8112

June 1981

FISHERIES RESEARCH INSTITUTE
College of Fisheries
University of Washington
Seattle, Washington 98195

ASSESSMENT OF PRODUCTION OF CHUM SALMON FRY
FROM THE BIG BEEF CREEK SPAWNING CHANNEL

by

Gary J. Duker and Michael W. Colley

ANNUAL REPORT
Anadromous Fish Project

Project No. AFC-97
Contract No. 80-ABD-UW1B
Project Period: October 1, 1979 to September 30, 1980

This project was financed with Anadromous Fish Act (P.L. 89-304)
funds through U.S. Department of Commerce, National Oceanic and
Atmospheric Administration, National Marine Fisheries Service.

Approved

Submitted: July 1, 1981

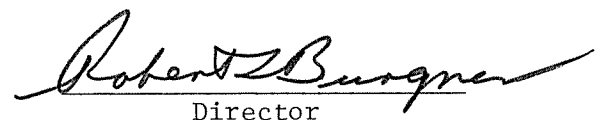

Director

TABLE OF CONTENTS

| | Page |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| Preface | vi |
| Acknowledgments | vii |
| Section I: The Influence of Water Velocity on Deposition and Egg-to-fry Survival of Chum Salmon in a Controlled-flow Environment . . by Gary J. Duker | 1 |
| Introduction | 1 |
| Methods | 2 |
| Experimental Sections | 2 |
| Measurements and Handling of Adult Chum Salmon | 3 |
| Monitoring of Incubation Environment | 3 |
| Chum Salmon Fry Production | 4 |
| Results | 5 |
| Observations on Adult Chum Salmon | 5 |
| Fecundity | 5 |
| Actual Egg Deposition | 5 |
| Fry Emergence | 5 |
| Egg-to-fry Survival | 5 |
| Pattern of Emergence | 6 |
| Coefficient of Condition | 6 |
| Environmental Parameters | 8 |
| Water Temperature | 8 |
| Dissolved Oxygen Concentration | 9 |
| Water Velocity | 9 |
| Quantity of Fine Sediment in the Test Sections | 9 |
| Discussion | 9 |
| Section II: Influences of Conspecific and Heterospecific Odor on Chum Salmon in a Controlled-flow Channel . . by Michael W. Colley and Gary J. Duker. . | 12 |
| Introduction | 12 |
| The Olfactory Hypothesis of Salmon Attraction | 12 |
| Materials and Methods | 13 |
| Results | 16 |

| | Page |
|----------------------------------------------------------------|------|
| A Solitary Female Chum Salmon as an Odor Source | 16 |
| A Solitary Male Chum Salmon as an Odor Source | 16 |
| Three Chum Salmon Males as an Odor Source | 16 |
| Three Coho Salmon Females as an Odor Source | 18 |
| Discussion | 18 |
| Olfactory Influences of Conspecific Salmon | 18 |
| Response to Females | 18 |
| Response to Males | 19 |
| Olfactory Influences of Heterospecific Salmon | 19 |
| Conclusions | 20 |
| Section III: An Examination of the Process of Species | |
| Recognition in Pacific Salmon . . by Gary J. Duker | 21 |
| Introduction | 21 |
| How Salmon Recognize Conspecifics: A Tentative Model | 21 |
| Experimental Design | 22 |
| Progress Report | 25 |
| Interspecific Interactions | 25 |
| Sensory Deprivation Experiments | 26 |
| Literature Cited | 28 |

LIST OF TABLES

| Table | Page |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| I-1. Coefficient of condition, K_D for fry emerging from the "fast" and "slow" test sections at five levels of emergence | 8 |
| II-1. Predicted responses by both sexes of chum salmon to odor cues broadcast by hetero- and conspecific Pacific salmon | 14 |
| II-2. Chi-square goodness-of-fit of data consisting of male and female chum salmon responses compared with an hypothesized response of 15:15 | 17 |
| III-1. The information each sensory system can provide a reproducing salmon about its potential mate or spawning environment | 23 |
| III-2. The types of spawning pairs that will be used to test the importance of visual and olfactory cues to adult chum salmon | 24 |

LIST OF FIGURES

| Figure | Page |
|--------------------------------------------------------------------------------------------------------------------------|------|
| I-1. Daily counts of chum salmon fry emerging from the "fast" and "slow" channel sections | 7 |
| II-1. A cross-sectional representation of the two-choice maze illustrating the key components of the apparatus | 15 |

PREFACE

The annual report from the Big Beef Creek Fish Research Station for 1979-1980 is divided in three sections. In the first section we report on the findings of an experiment designed to reduce silt deposition and increase egg-to-fry survival of chum salmon through control of water depth and velocity. Species recognition in Pacific salmon is the subject of the last two sections. In the second section we examine the potential influence of adult conspecific and heterospecific fish odors on the upstream migration behavior of chum salmon. A final report of this particular project is in preparation and will be available soon. In the third section we report on research in which we attempt to elucidate the sensory biology of adult chum salmon. Questions dealing with the importance of vision and olfaction during conspecific identification and spawning are presented and discussed.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the many people who were involved with various aspects of this project. First, special thanks must go to Messers. Mark Bedilion, Lynn McComas, Mike McDowell, Gary Maxwell, and Cliff Whitmus who put in many long hours collecting data, repairing equipment, and generally holding the project together. Mr. Steve Schroder helped design the study concerned with water velocity control, while Mr. Nic Bax provided considerable assistance during the analysis of data from this study. Mr. Bruce Snyder managed the budget, procured needed materials, and kept a watchful eye over the operation of this project.

Ms. Carol Sisley and Ms. Lila Stacey typed the rough drafts of the report while Ms. Dorothy Beall, Ms. April Richardson, and Mr. Marcus Duke prepared the final copy. Mr. Nic Bax and Drs. E. O. Salo and D. E. Rogers reviewed the manuscript and made many helpful comments.

Section I. The Influence of Water Velocity on Silt
Deposition and Egg-to-Fry Survival of Chum
Salmon in a Controlled-Flow Environment

by Gary J. Duker

INTRODUCTION

Chum salmon (Oncorhynchus keta) spawn in nests constructed in the gravel substrate of freshwater streams. There the offspring incubate until they emerge as fry and begin a seaward migration which will eventually bring them back as adults to the same stream 2, 3, or 4 years hence. Even though salmon have developed this use of gravel nests for the protection and development of their eggs, the greatest mortality occurs at this stage and normally exceeds 90% (Koski 1975). The main factors affecting embryonic (egg-to-fry) survival are: 1) the composition and stability of the gravel bed; 2) the quality and quantity of intragravel water; and 3) the density of spawners and redd superimposition. The success of spawning channels is dependent on the degree of control that man has exercised over these three factors.

In recent years, the egg-to-fry survival in the Big Beef Creek spawning channels has steadily decreased from ≈ 40 percent to ≈ 15 percent (1971 to 1977). This survival is still greater than that commonly experienced in the natural environment; however, the decrease in survival is a source of concern and indicates that our control over one or more of the crucial factors has declined. The stability of the gravel bed, optimal spawning density, and adequate flow of water into the channels have been maintained. Therefore, we must surmise that our control over the quality of the water entering the channels has declined. As an indication of this, the amount of fine materials removed during our annual cleaning of the gravel substrates in the channels has been increasing. We must conclude that the settling basins which were designed to collect suspended particles ("silt") from the water have become increasingly ineffective and now have decreased influence on the velocity of water moving to the channels; hence, suspended particles which were previously removed are now being carried to and deposited in the channels. The effectiveness of fines within a gravel substrate in reducing egg-to-fry survival has already been demonstrated in several of the spawning channels in use throughout the northwest (Koski 1975).

The current situation of the Big Beef Creek spawning channels, as well as at other northwest spawning channels, demands that effective techniques be devised to minimize the deposition of fine materials into the gravel bed. One potential strategy involves increasing the velocity of water moving through spawning areas within a channel in combination with the establishment of captive areas (i.e., small settling basins) where water movement slows appreciably and causes the deposition of suspended particles.

In the past, the Big Beef Creek channels have been managed to accommodate slight fluctuations in flow in order to avoid exposure of the incubation substrate to the atmosphere. Individual sections of the channels were consequently dammed to maintain an adequate water depth (5-7 cm). Unfortunately this damming also decreased the velocity of water moving through the channels; potentially allowing water-borne suspended particles to settle out.

To compare the two management strategies, an experiment was implemented to measure the deposition of fine sediments and egg-to-fry survival of chum salmon under different conditions of water velocity.

METHODS

Experimental Sections

Two parallel 24 x 3.0-m sections of the Big Beef Creek spawning channels separated by a water-tight wooden wall were used to examine the influence of water velocity on the egg-to-fry survival of chum salmon. The upstream ends of the two sections were dammed and modified to provide a common water source with equal flow for both sections. Water velocity and depth were regulated by removable dams (stoplogs) at the downstream end of the sections. For example, increasing the height of this dam raised the water depth in the section above it and decreased water velocity as the water flow remained the same.

Gravel was excavated from the upstream half of each section to repair (with 4 mil PVC sheeting) an underlying plastic membrane which had been placed in three sections in 1976 to prevent upwelling or seepage of water from under the channels (Duker 1977). The removed gravel and additional gravel of a similar size range (Duker 1977) quarried from the same location as the original gravel were then washed and mixed with a high-pressure water nozzle attached to a 300 gpm gasoline-powered water pump. Sufficient gravel was placed in the test sections to assure adequate depth for spawning by chum salmon (approximately 30 cm - K. Bruya, personal communication). The downstream half of each section was not treated in this manner. Salmon were not placed in this area as it was for flow regulation purposes only.

Each test section (upper 12.0 m) was divided figuratively into four 3.0 x 3.0-m experimental areas and each area was further subdivided into 100 30-cm squares using an overhead grid system made with string. Experimental areas were sampled for gravel composition prior to adult introduction and after fry emergence, as well as for dissolved oxygen content (D.O.) and temperature during the incubation stage. The 30-cm grid system provided the basis for the location of the gravel sampling sites and the D.O.-temperature standpipes.

Following the cleaning of the gravel and establishment of the grid system, a gravel sample was taken at a randomly-selected location within each 3 m x 3 m experimental area using a modified McNeil gravel sampler. As this experiment was designed to measure the effects of naturally deposited sediments (≤ 0.841 mm diameter), the initial gravel samples were examined to detect any difference in this critical size range between the two test sections.

The amount of materials 0.841 mm diameter and smaller did not exceed 1% of the total sample volume for any of the samples and no difference was apparent between the two test sections ($\bar{x}_1 = .447\%$ vs $\bar{x}_2 = .485\%$ for materials ≤ 0.841 mm diameter).

Eight PVC standpipes (2.54 cm in diameter) were buried in the two test sections, one per experimental area. The lower portion of the pipes was perforated with small holes that allowed the infusion of intra-gravel water which could be sampled for dissolved oxygen content and temperature.

Pickets were placed at the ends of the test sections to keep the fish in the area containing clean gravel.

Measurements and Handling of Adult Chum Salmon

Chum salmon were netted from a fish trap and placed in a fiberglass tank filled with about 400 liters of water containing MS222 (tricaine methanesulfonate) anesthetic within the recommended range of 1:12,500 - 1:25,000 (Bell 1964). Once anesthetized, the fork length and weight of each fish was recorded.

Experimental subjects in excellent condition were selected from the Big Beef Creek late-run of chum salmon over a 3-day period (December 6-8, 1979). We used the adult condition criteria established by Schroder (1973) and Koski (1975). Fish were tagged with large color-coded and numbered Peterson-type disc tags, and on December 8, 1979 the fish were trucked to the spawning channels where 7 males and 7 females were placed in each test section.

Fecundity of the females was estimated by a regression analysis of data on weight and total egg number collected from 31 late-run females during 1979. Daily observations were made on the location and activity of the females, and as fish died, they were removed from the test sections and females were examined for egg retention.

Monitoring of Incubation Environment

While chum salmon were in the test sections, water depth was maintained at ≈ 25 cm through the placement of additional stoplogs at the

downstream ends of the sections. After all spawning activities had ceased, these additional stoplogs, as well as the pickets, were removed and water depth in the test sections was reduced to ≈ 12 cm.

Before water depth was adjusted to create the "fast" and "slow" velocity sections, flows were sampled to determine whether there was a significant difference between the two sections. Water flow was measured 5 cm above the substrate with a Gurley meter at eight equidistant sample points across the upstream and downstream end of each test section. The Mann-Whitney test of the two-tailed null hypothesis that there was no difference between the flows was not rejected ($.20 > p > .10$).

With the assumption of equal flows, the experimental "fast" and "slow" velocities were obtained by removing a stoplog from the downstream end of the "fast" section but leaving the stoplog in place on the "slow" side.

Following adjustment of experimental velocities, dissolved oxygen content, water temperature and flows were sampled twice weekly. A dissolved oxygen-temperature meter (Yellow Springs Instrument Company, Model 54) with a probe modified for lowering into the standpipes was used to sample intragravel D.O.'s and temperatures. In addition, a Honeywell Brown type 602, 7-day, 2-probe continuous recorder was used to monitor the surface and intragravel water temperature of the spawning channel. Water flows were monitored in the manner described above.

Chum Salmon Fry Production

To assess survival, modified fyke net fry traps were placed at the end of the test sections to monitor the outmigrant fry (see Koski 1975 for description). The fry traps were checked each morning and all fry were removed.

Fry were measured routinely from each test section for length and weight. After fry were narcotized with MS222 they were measured (tip of snout to fork of tail) to the nearest mm and weighed with a Mettler P1200 balance. A maximum of 30 individual fry weights were recorded for each test section per day. The total number of fry emerging on a given day was then calculated by dividing the total group weight of fry by the average fry weight.

The fry were held in aerated tanks in the weir facility until night and then released near the outlet of the channels. The traps were removed after no fry had been trapped for a period of 7 days (May 18, 1980).

RESULTS

Observations on Adult Chum Salmon

Three days after introduction into the test sections (December 11, 1979), the females had established territories and had begun construction of their first nest. By December 16, it was apparent that all females had spawned completely and were merely guarding their redds. The following day, three dead females were recovered from the test sections and they retained a total of only 5 eggs. Unfortunately, a flood inundated the spawning channels during December 18-20, and the remaining females moved from the test sections; therefore, the number of eggs they retained could not be determined.

Fecundity

The regression equation for fecundity was determined by a regression analysis which assumed error in both the measurement of egg number and weight of the females. This equation was calculated using Bartlett's three-group method (Sokal and Rohlf 1969, p. 483-485) as there was no knowledge of the variances of x and y . The regression equation of fecundity (y) to \log_{10} body weight in kg (x) is

$$y = -6504.45 + 2729.90(x)$$

Actual Egg Deposition

The regression equation was used to predict the potential egg deposition (PED) for the individual females in the two test sections. This value does not reflect the actual egg deposition (AED) since females tend to retain some of their eggs. Since a count of actual egg retention was impossible for fish in this study, Koski's (1975) calculation of egg retention of approximately 5% of the estimated fecundity was used to estimate the actual egg deposition. The estimated total egg depositions were 22,211 in the "fast" section and 22,514 in the "slow" section. Survival estimates were based upon these predictions.

Fry Emergence

Egg-to-fry Survival

Production of fry from the two sections differed considerably, since 11,180 fry were captured at the end of the "fast" section and 5,150 fish were trapped from the "slow" section. Correspondingly, survival of fry from egg deposition to emergence differed significantly (50% survival in the "fast" section and 22% in the "slow" section).

Pattern of Emergence

Fish started to emerge in quantity (more than 2 per day) from the "fast" and "slow" test sections on the 12th and 17th of April 1980, respectively. The emergence of 50% of the fry was reached on April 26 for the "fast" section and April 27 for the "slow" section. For practical consideration, emergence ceased on May 9 for both sections (only one fish was captured after this date).

To increase precision, fry emerging between the 5th and 95th percentiles were assumed representative of the fry from each section and only these were used in comparisons. The middle 90% of the fry emerged in 15 days from the "fast" section and in 11 days from the "slow" section. This difference is illustrated by the graphs of emergence, where the "slow" section emergence pattern appears more peaked (Fig. I-1). A test for kurtosis was used (Snedecor and Cochran 1967, p. 87) to confirm this apparent divergence in pattern of emergence.

Peaked distributions show positive kurtosis and flat-topped distributions show negative kurtosis. The sample value (g_2) was positive for both curves; however, g_2 for the "fast" section was smaller than its standard deviation ($0.0136 < 0.0465$), so the amount of kurtosis was trivial. The sample value of 2.058 for the "slow" section was two orders of magnitude greater than its standard deviation (S.D. ≈ 0.068), and this indicates a more peaked distribution than the normal distribution.

Coefficient of Condition

Koski (1975) found that the size (both length and weight) of chum salmon fry, prior to feeding, is highly dependent upon their egg size. An index of fry size not dependent upon initial egg size is the coefficient of condition, K_D , developed by Bams (1970) where:

$$K_D = 10 \sqrt[3]{\text{weight (mg)/length (mm)}}$$

This index is a measure of the yolk absorption that has occurred. The value decreases as yolk reserves are used up. In this study, it was used to determine the stage of development at which fry began their downstream migration.

The average daily K_D s from the two test sections were tested for homoscedasticity by the F_{\max} -test (Sokal and Rohlf 1969, p. 371) and both sets of data were significantly heteroscedastic; consequently, non-parameteric tests were used to analyze the data. K_D factors were calculated from fry sampled at 10%, 25%, 50%, 75%, and 90% levels of emergence with 30 fish in each sample. There was a significant difference between K_D values for fish from the two test sections ($p < 0.001$). Examination of average K_D values at each level of emergence revealed that fish from the "slow" section consistently had the higher K_D values (Table I-1).

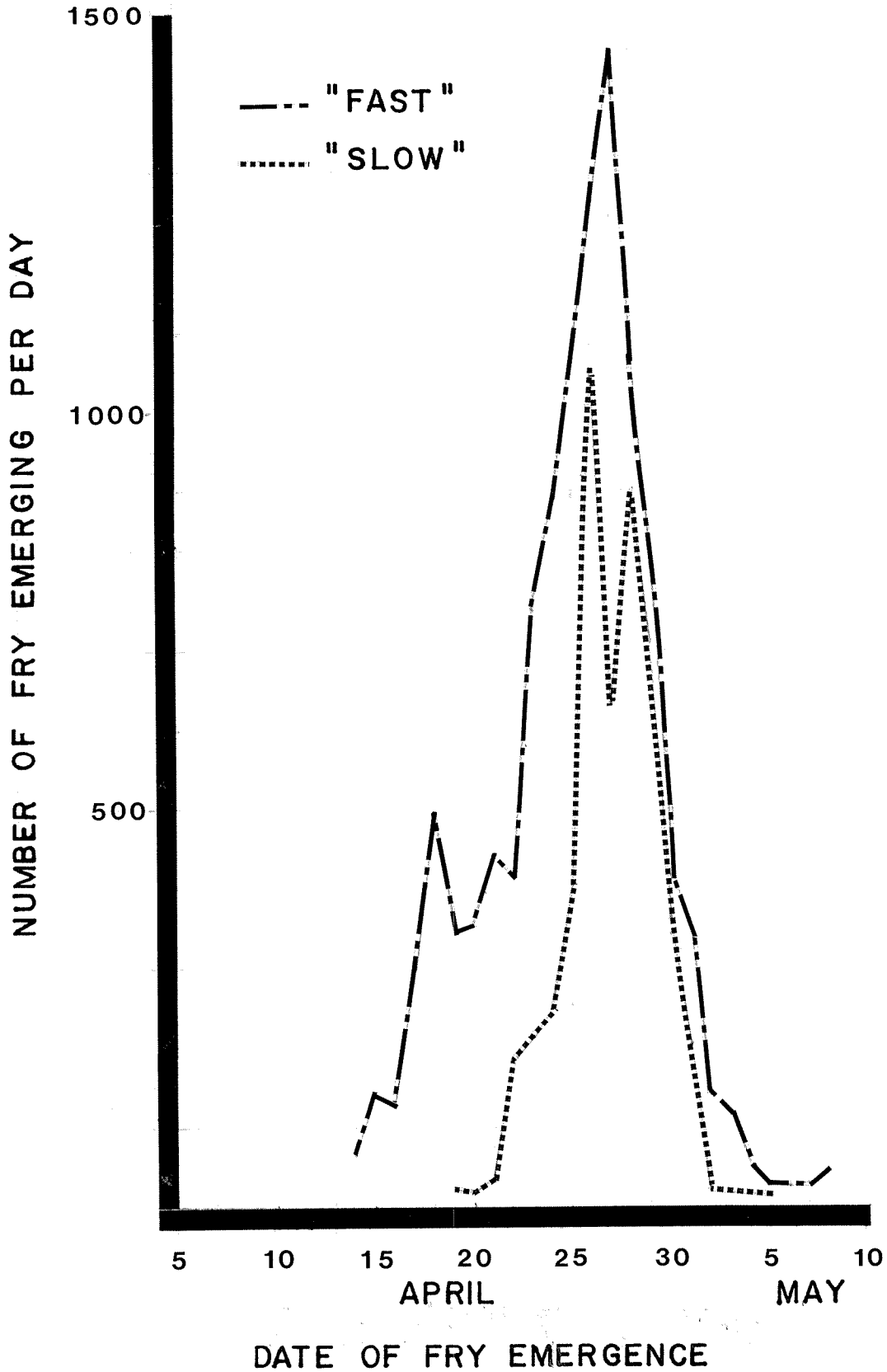


Fig.I-1. Daily counts of chum salmon fry emerging from the "fast" and "slow" channel sections.

Table I-1. Coefficient of condition, K_D for fry emerging from the "fast" and "slow" test sections at five levels of emergence (cumulative percentage).

Mean K_D at level of emergence (n = 30)

| | 10% | 25% | 50% | 75% | 90% |
|----------------|------|------|------|------|------|
| "Slow" section | 1.89 | 1.86 | 1.85 | 1.83 | 1.84 |
| "Fast" section | 1.86 | 1.83 | 1.81 | 1.83 | 1.82 |

The Kruskal-Wallis test was used to examine the K_D factors for differences in value for fry emerging within each section. This test showed significant differences between the fry within the "fast" section ($p < 0.001$), but not within the "slow" section ($.25 > p > .10$).

Nonparametric multiple comparison (Zar 1974, p. 156) of data from the "fast" section demonstrated significant difference in K_D values between the following emergence levels:

| | 10% | 25% | 75% | 90% | 50% |
|-------|-------------|-------------|-------------|-------------|-------------|
| K_D | <u>1.86</u> | <u>1.83</u> | <u>1.83</u> | <u>1.82</u> | <u>1.81</u> |

A common line under group means indicates that they have not been found significantly different ($\alpha = 0.05$).

Environmental Parameters

Water Temperature

Water temperature during the incubation period ranged from 10° to 20°C. The lowest temperatures were experienced at the end of January, well past the critical period of gastrulation when low temperatures (<4.4°C) can cause high mortality (for a review see Koski 1975).

Dissolved Oxygen Concentration

Some variation was observed among the eight sampling sites in the concentration of dissolved oxygen in the intragravel water. From spawning until the end of January, D.O.'s ranged from 3 mg/l to 15 mg/l. Unfortunately, failure of the sampling equipment shortly after the hatching of the alevins (late January) prevented further monitoring of D.O. concentrations. However, it is believed that the observed D.O. levels ($\bar{x}_F = 9.5$ mg/l for the "fast" section and $\bar{x}_S = 10.6$ mg/l for the "slow" section through January) remained adequate throughout the experimental period.

Water Velocity

While water velocity within each of the test sections varied somewhat, the relationship of the two sections did not change during the experimental period. Using the audible "click" produced by the Gurley meter during operation as our measure of velocity, we determined that the water velocity in the "fast" section averaged twice that of the "slow" section ($\bar{x}_F = 0.22$ m/sec vs $\bar{x}_S = 0.10$ m/sec for the downstream sampling locations).

Quantity of Fine Sediment in the Test Sections

The quantity of fine sediments (≤ 0.841 mm) within the gravel substrate increased dramatically during this study. Prior to the introduction of adult chum salmon, this fraction of the material represented less than 1% of the total gravel sample for all eight sampling locations; however, following fry emergence, it ranged from 15 to 61% of the total gravel sample. Partitioning this fraction into its smaller components revealed that the naturally deposited materials (≤ 0.210 mm) made up the largest part of the fraction, ranging from 85 to 99%.

Since the objective of the study was to examine the effect of water velocity on the deposition of fine sediments, the samples (materials ≤ 0.841 mm) from the two test sections were examined for a difference in composition using a two-sample t-test. First, the gravel samples from the two sections were tested for homoscedasticity using the variance ratio test (Zar 1974). There was insufficient evidence to conclude that the variances of the two sets of gravel samples were not the same. The t-test demonstrated that there was not a significant difference ($p > 0.50$) in the quantity of fine sediments between the "fast" and "slow" test sections.

DISCUSSION

Under natural conditions, chum salmon eggs and alevins are at the mercy of climatic conditions from the time of egg deposition to the emergence of the fry. Spawning channels control some of the abiotic, as well as biotic factors which contribute to the high mortality of salmon

during their freshwater incubation. However, the lack of success in controlling sedimentation of fine materials in many spawning channels has rendered these facilities practically useless.

Unfortunately, our experimental design to examine a strategy aimed at rectifying this problem suffered a major perturbation when the test sections were flooded just a few days after egg deposition. The amount of fine particles that were transported into the channels during the flood was atypical. In fact, when the water cleared and its level returned to normal after the flood, the incubation substrate in both test sections was covered with several centimeters of silt. This thick blanket of silt was reduced shortly after water velocity and depth were manipulated to experimental levels. It was not possible to measure the amount of material that was carried away by the water. Therefore, the flooding obscured any effects of velocity control in silt deposition.

The key observation from this study was: while certain abiotic factors (initial gravel composition, volume of flow, dissolved oxygen concentration during the first half of incubation, water temperature, and amount of fines in the gravel) were basically the same for both test sections, there were significant differences in egg-to-fry survival, fry condition, and pattern of fry emergence between the two test sections. Just how these differences are related to the one variable which was consistently different between test sections, water velocity, can be inferred from our understanding of the relationship of these fish to their environment.

To proceed we need to make one important assumption; the emergence of fry produced from seven females per section should mask any subtle differences in survival, timing, or condition of progeny from individual females. Therefore, any differences which are observed in these parameters should be related to the incubation environments.

In this study the time to the 5% level of emergence was 5 days greater for fish from the "slow" section. A delay in initial fry emergence has been linked to low dissolved oxygen concentration (Koski 1975). Koski found a reduction in intragravel dissolved oxygen content whenever the amount of fine sediment in the gravel exceeded $\approx 35\%$. While the level of fine particles in the test sections did not differ significantly, the amount of fines did exceed 35% for the "slow" side ($\bar{x}_S = 36\%$), but not for the "fast" section ($\bar{x}_F = 30\%$). This indicates that the dissolved oxygen content of intragravel water in the "slow" section may have exceeded the critical threshold in parts of the section.

The stage of development at emergence is generally of secondary importance to the timing of emergence, since it is crucial for the fry to emerge during periods of "optimum" condition. The possible consequences of emerging at the wrong time are adverse physical, as well as biological conditions in the marine environment. However, under adverse environmental conditions such as low dissolved oxygen or high amounts of

fines in the gravel, the stage of development takes precedence over timing (Koski 1975). Brannon (1965) and Koski (1975) have noted that continuous exposure to low dissolved oxygen can delay development and growth. Increased K_D values, such as recorded for fry emerging from the "slow" section when compared to fry from the "fast" section, are therefore an indication of a less than desirable environmental condition (Bruya 1981).

The interaction of fines in the incubation substrates and the differential velocities over these substrates undoubtedly are responsible for the observed variation in initial timing of emergence, and perhaps the stage of development of the emerging fry. However, the environmental agent responsible for this variation in survival can not be adequately described.

While this study was less than conclusive because of our inability to control flooding and to complete oxygen measurements, it did provide an indication of the value of maintaining maximum water velocity over an incubation substrate in a spawning channel.

Section II: Influences of Conspecific and Heterospecific
Odor on Chum Salmon in a Controlled-flow Channel

by Michael W. Colley and Gary J. Duker

INTRODUCTION

Although the role of olfaction in Pacific salmon (*Oncorhynchus* sp.) home stream migration is well documented, the role of olfaction in their reproductive biology is less fully understood. While active in their natal streams, adult salmon could influence the behavior of hetero- and conspecific adults by actively or passively releasing odors into the water. Thus, olfaction could play an important part in mate selection. This supposition is supported by the work of Newcombe and Hartman (1973), and Pete (1977) which suggests that olfactory attraction between sexually mature salmonids exists.

THE OLFATORY HYPOTHESIS OF SALMON ATTRACTION

Salo et al. (MS 1978) proposed that in Pacific salmon each sex exhibits distinct behavior during spawning activity. Males should search for females in an attempt to spawn with as many as possible, whereas females should devote their energies to finding a suitable location to bury their eggs.

An efficient mode of search by male salmon would be vital to minimize the potentially high energy cost of movement. Each unsuccessful search by a male salmon would reduce the amount of energy available for additional searches and for spawning. In light of this potentially limiting energy budget, a hypothesis was formulated where conspecific odor could be used by male Pacific salmon to efficiently locate potential mates. Conversely, female Pacific salmon, which do not actively seek mates, should not be influenced by conspecific odor.

In numerous streams of the Puget Sound Basin, Pacific salmon are found sympatrically. These species are not always temporarily or spatially separated, which could cause problems in the maintenance of genetic integrity. The avoidance or lack of response of male and female Pacific salmon to a heterospecific salmon odor is suggested as a possible mechanism preventing hybridization. Each species in effect would have its own chemical language that would distinguish it from other species.

This study examined the extent to which adult Pacific salmon respond to hetero- and conspecific odors. The experiment was designed to investigate chum salmon responses to

1. single male or female conspecific odor,
2. single female heterospecific odor,
3. odor from a group of three male or female conspecifics, or
4. a group of three female heterospecifics.

Chum salmon (*O. keta*) and coho salmon (*O. kisutch*) from the University of Washington's Big Beef Creek Research Station were used. Chum salmon were used as test subjects and the conspecific odor source. Coho salmon were used as the heterospecific odor source. The predicted reactions of chum salmon to conspecific and heterospecific odors are given in Table II-1.

MATERIALS AND METHODS

A two-choice maze, modeled after that of Newcombe and Hartman (1973), Fig. II-1) was constructed in the anterior section of one of the Big Beef Creek spawning channels (for details, see Colley 1981).

Once a fish or a group of three fish was selected for use as an odor source, it was placed in one of two holding tanks (solitary fish in a rectangular tank, multiple [three] fish in a larger circular tank) beside the channel and allowed to acclimatize--a minimum of one hour in the case of the nonterritorial males and overnight for the territory-seeking females. The tanks were supplied with water from a location upstream from the maze. A siphon pump was used to reduce the chances of contamination. This source was considered to be untainted by potential odors from spawning salmon. After passage through the tanks this water was used as the odor source. During each trial, water from the odor source tanks was released into one side of the maze (chosen at random), while untainted water, which had not passed through the tanks, was released into the other side at an equal rate to eliminate acoustic and flow differences.

To initiate a trial, a test fish of the desired species and sex was selected at random from a holding area and placed in the holding pen of the maze, downstream from the odor source. Each fish was allowed 10 min to acclimate to the test apparatus. After this period the holding pen was opened and each test fish was allowed one hour, measured from the time it exited from the pen, to swim upstream into one of the arms of the maze. The time between exiting from the pen and entering into the trap in either arm was recorded. If after one hour the test fish did not leave the pen or failed to make a selection, the trial was terminated and scored as no trial. Similarly, a trial was scored as no trial if the test fish bolted from the trap. Test fish were utilized only once to preclude influences from having previously experienced the maze (for further detail, see Colley 1981).

A chi-square goodness-of-fit test was used to test the null hypothesis that the behavior of test fish would not be affected (1:1 = accept: reject) by hetero- and conspecific odors. The null hypothesis was rejected when the calculated chi-square value exceeded the critical value of $\chi^2 = 3.841$ (Zar 1974) for one degree of freedom at a significance level (α) of 0.05.

Table II-1. Predicted responses by both sexes of chum salmon to odor cues broadcast by hetero- and conspecific Pacific salmon.

| Sending individual | Receiving individual | Predicted response of receiving individual |
|-----------------------|----------------------|--------------------------------------------|
| <u>Conspecific</u> | | |
| Female chum | Male chum | Search for sending individual |
| Female chum | Female chum | Random response |
| Male chum | Male chum | Random response |
| Male chum | Female chum | Random response |
| <u>Heterospecific</u> | | |
| Female coho | Male chum | Random response or avoidance |
| Female coho | Female chum | Random response or avoidance |

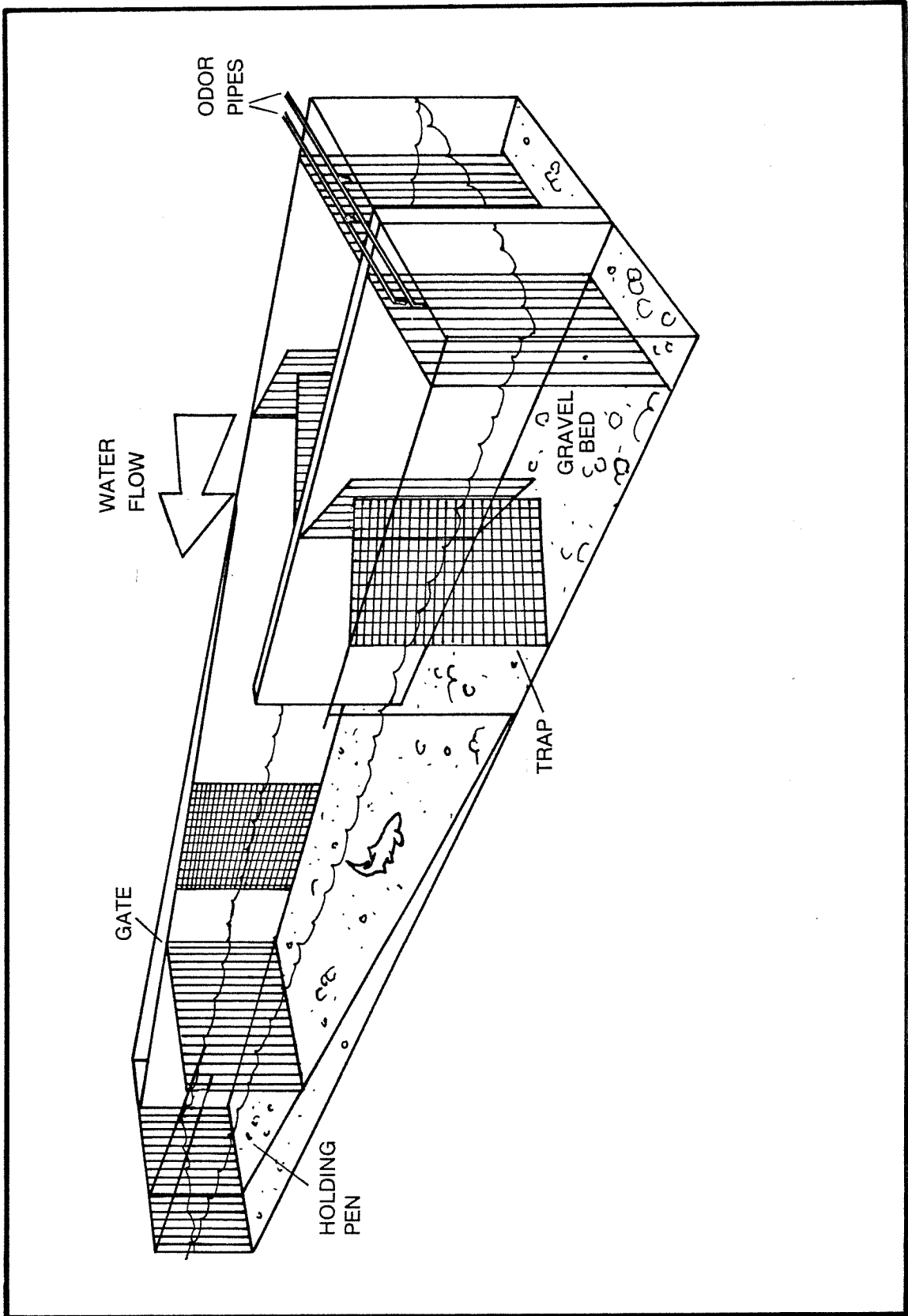


Fig. II-1. A cross-sectional representation of the two-choice maze illustrating the key components of the apparatus.

RESULTS

A Solitary Female Chum Salmon as an Odor Source

Tests with male and female chum salmon as the test subjects were conducted to see if water passing a conspecific female influenced response in the two-choice maze. The null hypothesis that test fish would not respond to possible odors from a single female chum salmon was not rejected for either sex (Table II-2).

A Solitary Male Chum Salmon as an Odor Source

Male and female chum salmon were tested for response to possible odors from a solitary male chum salmon. The hypothesis that choice of arm in the maze was not affected by the presence of a male chum salmon in the water source was not rejected (Table II-2).

In the tests using females as test fish, one odor-source fish remained extremely stressed during the duration of the test. As a result of its state, the last 10 tests using this fish were not used in calculating the chi-square statistic.

A Solitary Female Coho Salmon as an Odor Source

To examine the effect of a heterospecific odor source, the responses of male and female chum salmon to possible odors from a single coho female were tested. Female response was not significantly different from the proposed 15:15 ratio (Table II-2). Conversely, male chum response to female coho odor showed a significant departure from the hypothesized 15:15 ratio.

Three Chum Salmon Females as an Odor Source

The null hypothesis that male or female chum salmon would not respond to possible odors from a group of three female chum salmon was not rejected (Table II-2).

Three Chum Salmon Males as an Odor Source

Both male and female chum salmon exhibited no significant response to possible odors from three male chum salmon. Chi-square values for both tests were computed to be 0.533 (Table II-2), far below the critical chi-square value.

Table II-2. Chi-square goodness-of-fit of data consisting of male and female chum salmon responses compared with an hypothesized response of 15:15 (accept/reject).

| Test fish | Number tested | No. tests not used | Choice | | Chi-square | Conclusion |
|-------------------------------------------------|---------------|--------------------|--------|--------|------------|---------------|
| | | | Accept | Reject | | |
| A solitary female chum salmon as an odor source | | | | | | |
| Male chum | 39 | 9 | 19 | 11 | 2.133 | Do not reject |
| Female chum | 39 | 9 | 20 | 10 | 3.333 | Do not reject |
| A solitary male chum salmon as an odor source | | | | | | |
| Male chum | 40 | 10 | 16 | 14 | 0.133 | Do not reject |
| Female chum | 33 | 13* | 12 | 8 | 0.800 | Do not reject |
| A solitary female coho salmon as an odor source | | | | | | |
| Male chum | 34 | 4 | 9 | 21 | 4.800 | Reject |
| Female chum | 33 | 3 | 19 | 11 | 2.133 | Do not reject |
| Three chum salmon females as an odor source | | | | | | |
| Male chum | 38 | 8 | 16 | 14 | 0.133 | Do not reject |
| Female chum | 30 | 0 | 14 | 16 | 0.133 | Do not reject |
| Three chum salmon males as an odor source | | | | | | |
| Male chum | 31 | 1 | 17 | 13 | 0.533 | Do not reject |
| Female chum | 33 | 3 | 17 | 13 | 0.533 | Do not reject |
| Three coho salmon females as an odor source | | | | | | |
| Male chum | 33 | 3 | 7 | 23 | 8.533 | Reject |
| Female chum | 33 | 3 | 16 | 14 | 0.133 | Do not reject |

* Only 20 tests valid, hypothesized response 10:10.

Three Coho Salmon Females as an Odor Source

Male chum salmon response showed a significant departure from the 15:15 ratio so that the null hypothesis was rejected (Table II-2). Female response showed no significant deviation from the expected 15:15 ratio.

DISCUSSION

In this study, no individual test revealed a significant response by chum salmon to odors from conspecifics, and only chum salmon males exhibited a response to the heterospecific coho salmon. The lack of a significant response suggests that the olfactory system in chum salmon could be of secondary importance to other sensory systems, or that the hypothesized species-specific odors do not exist, or that the experimental design was inadequate because of odor dilution or volatility.

Olfactory Influences of Conspecific Salmon

Response to Females

When the response of male and female chum salmon to a single female as an odor source was examined, it was found that there was a tendency for both sexes to be attracted by the water source containing the female chum salmon, although neither response was statistically significant using these procedures.

When male chum salmon were tested against water from the tank containing three chum salmon females, there was no tendency toward attraction. This contradicted the expected response that males would be attracted to the odor of conspecific females as potential mates. This conflicts with the apparent response to a single female odor source and casts doubt on the suggested response by males to a female odor source.

The suggestion of an attraction of female chum salmon to conspecific female odor deviated from the predicted lack of response. The tendency of females to show preference for water containing other females may indicate that they use these conspecific odors during the search for suitable spawning habitat. Thus, they could save time and energy by moving directly toward a conspecific female.

There was no apparent tendency by females to move toward the multiple odor source. This behavior was consistent with the theory that females would not respond to odor since their primary goal would be to find a suitable spawning site (Schroder 1973, Salo et al. MS 1978). Since this response was different from responses to a single odor source, it was possible that confinement of the groups of fish in the circular tank caused some change in the composition of odors in the water source.

Perhaps crowding produced an odor change that inhibited an attractive response. The same reasoning would explain the difference in response of male chum salmon to single and multiple female odor sources.

Response to Males

Male and female chum salmon's response to a water source containing a solitary male showed no attraction to the potential odor source.

In one set of trials (not included in the results), there was a high incidence of rejection (1:8, accept : reject) by female chum salmon to the odor source water. It is believed that the male used in this set of trials was stressed by the tank used to hold odor source fish. Perhaps an unknown type of fright substance was emitted by the male because of his confinement.

When male and female chum salmon were tested against groups of three male chum salmon, neither sex showed an attraction. The model predicts that regardless of the odor concentration there should be no attraction to male salmon. Males should have no need to find other males since they cannot spawn with them, and the presence of other males would not guarantee the presence of females. Conversely, benefit could be derived by a female that searches for a male as a potential mate; however, the net cost in terms of time lost and energy expended that could be used for nest site exploration and construction would be prohibitive.

Olfactory Influences of Heterospecific Salmon

In these tests, female chum salmon showed a tendency to move toward a single female coho salmon as an odor source, but displayed random behavior in response to a multiple odor source. Perhaps, as with female chum salmon odor, a single odor source signals to the approaching female that there is available spawning habitat ahead, and a multiple odor source reveals competition upstream. Alternatively, the experimental design may have elicited an unknown "fright odor" from the group of three females.

In the only significant trials, male chum salmon chose to avoid the arm of the maze where water from the tanks containing single and groups of coho females was introduced. This behavior was expected since a random response or avoidance was predicted. Since male chum salmon tend to be attracted toward female chum but repelled by female coho salmon, one could conclude that chemical separation is present in chum and coho salmon interactions. Since males are actively seeking a mate, an olfactory isolating mechanism could act on males to avoid heterospecific females.

CONCLUSIONS

These results suggest that olfaction does not play a major role in the guidance of salmon to a potential mate. This was illustrated in the lack of a significant response of male and female chum salmon to conspecific odors.

Responses to heterospecific odor revealed a potential olfactory isolating mechanism operating on the male. Since males actively seek a mate and females do not, an isolating mechanism could work on the male to inhibit heterospecific pairing.

Section III: An Examination of the Process of Species
Recognition in Pacific Salmon

by Gary J. Duker

INTRODUCTION

There are five closely related species of Pacific salmon native to North America. These are the chum, Oncorhynchus keta; sockeye, O. nerka; pink, O. gorbuscha; coho, O. kisutch; and chinook, O. tshawytscha, salmon. All occur sympatrically and because very few natural hybrids have been observed, it appears that reproductive isolating mechanisms must be present.

To date, no studies have been made to systematically determine which isolating mechanisms may be used by salmon to avoid genetic introgression. Muller (1942) and Mayr (1963) have proposed that the genetic integrity of a species can be maintained by: 1) geographic or spatial isolation, including different habitat preferences or breeding seasons, 2) mechanical isolation caused by morphological differences in genitalia, 3) gamete, zygote or hybrid mortality, or sterility, and 4) ethological isolation.

A review of the reproductive biology of Pacific salmon suggests that genetic integrity of each species is maintained by ethological isolating mechanisms. These are loosely comprised to behavioral activities and physical characteristics which lead to species recognition.

The objective of this research is to determine what sensory systems are being used and which physical characteristics, for example color, shape, and odor, or behavioral activities, such as courtship movements and periods of sexual activity, are important in the choice of conspecific mates by salmon.

HOW SALMON RECOGNIZE CONSPECIFICS:
A TENTATIVE MODEL

The process of species recognition is analogous to the use of a taxonomic key, with the animals receiving information ("cues") regarding the desirability of any potential mate based upon the presence or absence of certain characteristics.

The cues used by Pacific salmon to choose conspecific mates are divisible into two basic categories, those which can be transmitted and detected without (long-range) and with (short-range) visual contact. Long-range cues include sounds and odors. Short-range cues consists of visual patterns based upon movement, body shape, and placement of pigments, physical contact, as well as odors and sounds.

For successful conspecific spawning to occur four pieces of information are necessary, namely: the species, sex, reproductive status,

and exact location of the potential mate, and this information can only be provided by short-range cues.

Experiments performed by Duker (1977) and others suggest that each sensory system (visual, olfactory, auditory, and tactile) may be capable of determining the desirability of potential mates. (The involvement of these four sensory systems in the various stages of Pacific salmon reproductive behavior is summarized in Table III-1). However, the information needed for species recognition can only be detected visually or through short-range olfactory cues. The auditory and tactile sensory systems may supply information but not enough to ensure that a mate is a conspecific.

EXPERIMENTAL DESIGN

The model makes a variety of predictions that can be tested. Research has been limited at present to an examination of the predictions which deal with species recognition at the short-range level.

The prediction that conspecifics are preferred as mates was tested by observing whether salmon persistently chose conspecific mates under a variety of conditions. Heterospecific pairs of coho, chinook, chum and pink salmon were used to examine the role of species-specific physical characteristics and behavioral activities in the selection of mates.

The importance of short-range cues in the species recognition process was examined by arranging pairs of chum salmon where one or both members have been deprived of visual and/or olfactory information. Visual cues were blocked by placing opaque or translucent patches over the eyes effectively blinding the animal, or made fallacious by changing body color patterns through the use of colored stockings and dyes. A method that would disguise olfactory cues has not been found, but fish were rendered anosmic by injecting a modified wax into their olfactory sacs. With these techniques, the validity of the prediction was tested by observing whether pairs with the particular sensory deficiency or falsified information were able to spawn. The treatments and predictions are summarized in Table III-2.

Since detailed observations on each spawning pair were required, the viewing chamber located at the anterior end of the Big Beef Creek controlled-flow stream was used for all experimental trials. This chamber allows underwater viewing of the test pairs without disturbing them.

Table III-2. The types of spawning pairs that will be used to test the importance of visual and olfactory cues to adult chum salmon.

| Type of spawning pair Fish A | Type of spawning pair Fish B | Sensory deficiency experienced by Fish A | Model prediction |
|---------------------------------|---------------------------------|------------------------------------------------------------------------|-------------------------------------------------------------------------|
| Blinded | Unaltered | Complete lack of visual information. | Will not spawn. |
| Anosmic | Unaltered | Lack of olfactory information. | Will spawn - vision supplies sufficient information. |
| Anosmic | Body and mouth colors altered | Reduction of olfactory and visual information | Will not spawn. |
| Unaltered | Body and mouth colors altered | Reduced visual information | Will spawn - olfaction supplies sufficient information |
| Anosmic | Body color altered | Lack of olfactory information, partial reduction of visual information | Will spawn because mouth color provides a species-specific cue |
| Anosmic | Mouth color altered | Lack of olfactory information, partial reduction of visual information | Will spawn because body color provides an adequate species-specific cue |

PROGRESS REPORT

Interspecific Interactions

Reports of social interactions between heterospecific Pacific salmon on spawning grounds are rare and descriptions of such interactions are rarer. Moreover, the importance of these interactions on the nature of isolating mechanisms and species recognition has been largely overlooked. The value of observing such interaction is straightforward. Just as differences in the expression of behavior among conspecifics communicates something about the individuals, the interaction between heterospecific communicates something about each species. By comparing the interactions of conspecifics and closely-related heterospecifics, it becomes possible to describe the types and methods of information exchange used in species recognition.

In each heterospecific trial, a sexually-mature female was released into the viewing chamber and left to construct a nest. When there was evidence of active nest construction, a male was introduced. At the onset of male activity, usually courtship movements directed toward the female, the female responded by chasing the male from the nest area. Early interaction between fish was dominated by male courtship movements and female aggression (i.e., chasing and biting). The female's aggressive behavior rapidly waned and within two hours aggressive responses were rarely observed. During this time period, a noticeable change was also observed in the 'nest-related' behavior of the female. Activity which would normally be directed toward the nest (i.e., digging, probing, and crouching) became misdirected toward the gravel areas adjacent to the nest within the enclosure. This was generally followed by a period of reduced activity or by a cessation of all activity, where the female rested on or near the substrate with minimal movement. During this time, the male was able to maintain a position beside the female, as well as to attempt to court her when she moved without reprisal.

These observations have provided several valuable insights into the process of conspecific recognition in Pacific salmon. First of all, qualitative and quantitative differences in courtship movements are not readily apparent for males of the four species. Whether slight (but crucial) differences in the frequency or execution of these activities may serve as species-specific cues has not been demonstrated and may be difficult to establish considering the individual variation that was observed.

Males showed little reluctance in courting heterospecific females. While one might expect males to be less discriminating than females, the complete lack of any avoidance reactions in these trials poses some questions about the discriminatory abilities of the male Pacific salmon. If one were to attempt to fit these observations to a model of conspecific recognition, one could have two quite distinct patterns based on the sex

of the individual. Males could be characterized as searching for some general female salmon form and activity and not discriminating the color or placement of pigments or other species-specific cues until some crucial stage in their spawning behavior if at all. Females, on the other hand, appear to respond immediately to the species characteristics of males. Body shape, color and placement of pigments and other components, such as odor, allow immediate recognition with no mistakes.

Finally, the misdirected behaviors of the females would appear to be an effective method of increasing the likelihood of the appearance of a conspecific mate. The activity could generate long-range cues and reduce the courtship activity of an unwanted heterospecific male.

Sensory Deprivation Experiments

Through sensory manipulation, Duker (1977) demonstrated that nest site selection by female chum salmon was dependent on visual, as well as, olfactory and tactile, input from the fish' environment (Table III-1). These techniques and the resulting observations opened the door to a multitude of questions concerning the sensory biology of Pacific salmon.

At the beginning of this research, we did not know which phenotypic traits or sensory systems were being used in the recognition process, or whether they were the same for each sex or species. In response to this general paucity of information, several basic hypotheses based on the predictions in Table 2 were formed and tested. Following these trials, new questions and test situations based on the answers to the previous hypotheses were formed and tested.

The initial tests demonstrated that form vision was essential for the successful spawning of chum salmon. Apparently, males are dependent on vision supplying the information which determines the exact location of a potential mate, as well as her reproductive status. As evidence of this, courtship activities of the male were frequently misdirected and males failed to locate or recognize sexually active (i.e., crouching and gaping) females. What is interesting here is the fact that the males did exhibit typical courtship movements even though they suffered from some form of visual impairment. The previous observations on heterospecific pairs demonstrated the male "quivering" is not in response to species-specific information, but appears to be a general response to some general female-transmitted information.

In response to these observations, blinded males were paired with unaltered heterospecific females and with blinded conspecific males. The blinded males attempted to court the heterospecific females, but they did not exhibit courtship movements when paired together. Therefore, males are able to discriminate between males and females utilizing generalized information which is not species-specific and not due to

vision. There appear to be three potential routes for information transmission: 1) olfactory-with females releasing chemical signals, 2) auditory-based upon females' digging or biological sounds, or 3) tactile.

Of these three routes, tactile would appear to be the least likely because these fish rarely came in contact while interacting and impaired males were observed "quivering" before contact was made with a female. While the auditory route would appear to be a potential channel to use for information transmission, the nature of the signals, as well as the physiology of this particular sensory system make it a difficult route to explore. As a result, work was concentrated on the olfactory system of the male, especially after earlier research (Duker 1977) demonstrated a complex relationship between olfaction and female behavior.

Unlike anosmic females, especially solitary ones which exhibited little nest selection behavior and no nest construction, anosmic males exhibited no changes in behavior. These observations have demonstrated that the importance of olfaction differs with the sex of the fish.

To proceed further with this line of questioning, we were faced with the dilemma of having to simultaneously manipulate the visual and olfactory sensory systems of the chum salmon male. To avoid any potential confounding effects, a technique of altering what the male sees in combination with olfactory occlusion was used.

These trials with visibly-altered females and anosmic males revealed that visual and olfactory species-specific information is not necessary to stimulate male activity. In conclusion, males appear to be responding to a general female pattern (form and shape, as well as activity) which may be primarily visual in constitution.

The previous section revealed that a Pacific salmon female when confronted with a heterospecific male will first become aggressive and then inactive. It was hypothesized that the females were responding to the visual image (i.e., external body coloration) of the males. To test this hypothesis, the body coloration of conspecific males was altered and each male was paired with an active female. The females in these trials were aggressive toward the altered males initially which suggests that male body coloration plays an important role in the female's recognition of a conspecific mate. However, female aggression was short in duration and the trials ended with the fish spawning. Thus, the females were able to successfully identify the males by means other than species-specific body coloration.

LITERATURE CITED

- Bams, R. A. 1970. Evaluation of a revised hatchery method tested on pink and chum salmon fry. *J. Fish. Res. Board Can.* 27:1429-1452.
- Bell, G. R. 1964. A guide to the properties, characteristics, and uses of some general anaesthetics for fish. *Fish. Res. Board Can. Bull.* No. 148. 4 pp.
- Brannon, E. L. 1965. The influence of physical factors on the development and weight of sockeye salmon embryos and alevins. *Inter. Pacific Salmon Fish. Comm., Prog. Rep. No. 12.* 26 pp.
- Bruya, K. J. 1981. The use of different gravel depths to enhance the spawning of chum salmon, *Oncorhynchus keta*. M.S. Thesis, Univ. Washington, Seattle. 86 pp.
- Colley, M. W. 1981. Influences of conspecific and heterospecific odor on chum salmon (*Oncorhynchus keta*) in a controlled-flow channel. M.S. Thesis, Univ. Washington, Seattle. 69 pp.
- Duker, G. J. 1977. Nest site selection by chum salmon (*Oncorhynchus keta*) in a spawning channel. M.S. Thesis, Univ. Washington, Seattle. 113 pp.
- Koski, K V. 1975. The survival and fitness of two stocks of chum salmon (*Oncorhynchus keta*) from egg deposition to emergence in a controlled-stream environment at Big Beef Creek. Ph.D. Dissertation, Univ. Washington, Seattle. 212 pp.
- Mayr, E. 1963. *Animal species and evolution.* Harvard Univ. Press, Cambridge. 797 pp.
- Muller, H. J. 1942. Isolating mechanisms, evolution, and temperature. *Biol. Symp.* 6:71-125.
- Newcombe, C., and G. Hartman. 1973. Some chemical signals in the spawning behavior of rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.* 30:995-997.
- 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, Univ. Washington, Seattle. 55 pp.
- Salo, E. O., E. L. Brannon, W. K. Hershberger, S. L. Schroder, and G. J. Duker. 1978. Ethological isolating mechanisms and gene flow in two species of Pacific salmon. Univ. Washington, Fish. Res. Inst. Research proposal submitted to the National Science Foundation. Unpublished. 78 pp.

- Schroder, S. L. 1973. Effects of density on the spawning success of chum salmon (Oncorhynchus keta) in an artificial spawning channel. M.S. Thesis, Univ. Washington, Seattle. 78 pp.
- Snedecor, G. W., and W. G. Cochran. 1967. Statistical methods. Iowa State Univ. Press, Ames. 590 pp.
- Sokal, R. R., and F. J. Rohlf. 1969. Biometry. The principles and practices of statistics in biological research. W. H. Freeman and Co., San Francisco. 776 pp.
- Zar, J. 1974. Biostatistical analysis. Prentice-Hall, Inc., Englewood Cliffs, New Jersey. 620 pp.