CONTROL OF VISUAL PIGMENT PROPORTIONS IN TWO ANADROMOUS FISHES

by

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FOREWORD

This dissertation is organized into self-contained chapters. A shorter version of Chapter II has been published (Gen. Comp. Endocrinol. 23, 58-62; 1974); Chapter III is in press (Vision Research); and Chapter IV has been submitted to General and Comparative Endocrinology. Chapter V will be submitted to Canadian Journal of Zoology. Chapter I is an overall introduction to the dissertation, but further specific introductory material is given in each of the following chapters. The emphasis in Chapter I is on background information given less emphasis in the other chapters, but which is helpful for a general understanding of the dissertation.

CHAPTER I

INTRODUCTION

A vertebrate visual pigment consists of a protein moiety (opsin) and a chromophore (retinene, which is the aldehyde of vitamin A). The pigments usually found in retinal extracts are from the rods and hence used for scotopic vision, but with favorable species cone pigments can also be extracted (see Munz and McFarland, 1975). Only the rod pigments will be considered in this dissertation.

Experimental work on the vertebrate visual pigments began with the studies by Kühne in the late nineteenth century (Kühne, 1878). Köttgen and Abelsdorff (1896) extracted pigments from the retinae of 4 mammals, 1 bird, 3 amphibians, and 8 freshwater fishes and studied these pigments with a crude spectrophotometer. The pigments of the mammals, bird, and amphibians all had absorbance maxima (λ_{max}'s) near 500 nm, and the pigments of the freshwater fishes had maxima near 540 nm.

In the 1930's Wald demonstrated that the chromophore of visual pigments was vitamin A aldehyde, and furthermore, that there were two kinds of vitamin A and hence two kinds of retinene, which he called vitamins A_1 and A_2 and $retinene_1$ and $retinene_2$, after Edisbury *et al.* (1937; see Wald, 1946a, or Dartnall, 1957, for detailed references to information in this and the following two paragraphs). Also, the difference between the two types of pigments seen by Köttgen and Abelsdorff was due to the type of chromophore: pigments of mammals, birds, and amphibians having $retinene_1$ and pigments of freshwater fishes

having retinene$_2$. Wald retained the older term rhodopsin for the red-colored, retinene$_1$-based pigment with λ_{max} at 500 $\pm$ 2 nm and introduced the term porphyropsin for the purple-colored, retinene$_2$-based pigment with λ_{max} at 522 $\pm$ 2 nm (Wald's revision of the 540 value reported by Köttgen and Abelsdorff).

Wald then surveyed other vertebrates, studying the ocular vitamins A in some species and the visual pigments in others. He reported that the eyes of marine fishes contained vitamin A_1 or rhodopsin, unlike the freshwater fishes; and that diadromous and euryhaline fishes had vitamin A_1 (or rhodopsin) alone, vitamin A_2 (or porphyropsin) alone, or mixtures of vitamins A_1 and A_2 (or rhodopsin and porphyropsin), with the "pigment of the spawning habitat" either alone or predominating. He also found that larvae of the bullfrog (Rana catesbeiana) had vitamin A_2 in their eye tissues, whereas the adults had vitamin A_1 and rhodopsin. The reptiles, which are generally deficient in rods, were not investigated at this time. The eyes of a handful of reptiles, studied later, contained vitamin A_1, with the exception of two freshwater turtles, which had A_2 (see Wald, 1963).

Wald attempted to make phylogenetic sense from this distribution, and he emphasized the movement from hydrating to dehydrating environments during evolution. In his scheme, (primitive) freshwater vertebrates have porphyropsin, land and marine vertebrates have rhodopsin, and diadromous and euryhaline fishes have the pigment of the spawning habitat exclusively or predominantly. The change from porphyropsin to rhodopsin in bullfrogs during metamorphosis was viewed as a recapitulation. Wald considered that the difference in visual function between rhodopsin and

porphyropsin was trivial and therefore not subject to selection (Wald, 1963, p. 20).

Wald's hypothesis was criticized on two major grounds: 1) Dartnall (1957, 1962) criticized the methodology used by Wald (the use of the indirect vitamin A test in some cases, and failure to test the homogeneity of visual pigment extracts in the others) and the incompleteness of the survey (e.g., the few exceptions among the freshwater fishes, reported by other workers and discounted by Wald (1960a, p. 324), comprised a substantial fraction of the total number of species properly examined); and 2) Simpson (1964) criticized the interpretation itself. Simpson wrote (p. 1537):

> He [Wald, 1963] finds it inexplicable and almost an unnecessary complication that, for instance, reptiles, primitively having A_1 [i.e., vitamins A_1 in the eye tissues], 'revert' to A_2 when they adapt to fresh water [freshwater turtles]. To an organismal biologist, the picture, including the apparent anomalies and supposed reversions, suggests interpretation in terms of adaptation, primarily, and phylogeny only secondarily. Many, but perhaps not quite all, of the observations would be explained if we assumed that A_2 is adaptive in freshwater forms and A_1 in land and saltwater forms--so much so that selection usually produced these adaptations rapidly and tended to erase purely phylogenetic effects. I have no idea what the difference might be, but suggest that study from this point of view might clarify the molecular function involved.

Because of work during the last 20 years (see below), Wald's hypothesis has been discarded by visual physiologists, but it has been slow to die outside the field (e.g., Crim, 1975, p. 233), probably because of the numerous reviews by Wald (e.g., 1946a, 1949, 1952, 1958, 1960a, 1963), its appearance in textbooks, and the time required for its replacement with a new theory, convincing and documented.

It has been suggested that vitamin A_2 might serve an osmotic function in freshwater vertebrates (Willmer, 1956). However, no such function has been found. Furthermore, there is no consistent relationship between liver and ocular stores of vitamins A_1 and A_2. For example, the carp (Cyprinius carpio) has vitamin A_2 and porphyropsin alone in the eyes, but a mixture of 65-75% A_1 and 25-35% A_2 in the liver (Wald, 1939; Crescitelli and Dartnall, 1954). In addition, the generalization that freshwater fishes have only porphyropsin has been overturned. In a large survey, Schwanzara (1967) found that the number of species of purely freshwater fishes with porphyropsin alone is equalled by the number having mixtures of rhodopsin and porphyropsin, and some species may have rhodopsin alone.

Other generalizations in Wald's hypothesis have also been overturned with new evidence. The proportions of rhodopsin and porphyropsin in diadromous fishes such as salmon are not static, but can change from one extreme to the other during the life cycle (Beatty, 1966). As mentioned above, freshwater fishes may also have mixtures of the two pigments, and the proportions can change seasonally (Dartnall et al., 1961; and see Allen et al., 1973). Moreover, the generalization that rhodopsin and porphyropsin always have absorbance maxima at 500 $\pm$ 2 nm and 522 $\pm$ 2 nm respectively, has been proven untrue. Retinene$_1$-based rod visual pigments vary from the golden-colored pigments of deep-sea fishes to the purple-colored pigments of geckoes (λ_{max} range from 467 to 530 nm; see Lythgoe, 1972b); and retinene$_2$-based pigments are also diverse in λ_{max}--the absorbance maxima are at longer wavelengths on the average, but the range overlaps that of the retinene$_1$-based pigments. The terms

rhodopsin and porphyropsin have been retained for the rod pigments, but they have been redefined to indicate the type of chromophore only. (Other terminology commonly used today is to give the λ_{max} of the pigment with the subscript "1" or "2" to indicate the type of chromophore. Thus $VP503_1$ (or $P503_1$) and $VP527_2$ (or $P527_2$) refer to the rhodopsin and porphyropsin in several salmonids (Munz and Beatty, 1965). This example is a "pigment pair," i.e., both pigments are based on the same opsin, so that the rhodopsin and porphyropsin are interconvertible by changing the chromophore.)

With additional data the generalizations that mammals, birds, reptiles, and marine fishes have only rhodopsins have proven to be good, with only a few exceptions (see Lythgoe, 1972b, and Munz and McFarland, 1973). The data for amphibians are too complex and too few to make any sense at present (see Bridges, 1972).

What then is the difference in function between rhodopsins and porphyropsins and the reason for the diversity in absorbance maxima in each class? Modern workers have focused on differences in visual function, and work has centered on fishes, because the differences in both pigments and photic environments are largest among these vertebrates. The greatest success has been achieved in studying the rhodopsins of marine fishes. Two main hypotheses have been advanced: the Sensitivity Hypothesis of Munz (1958b, 1964, 1965) and the Contrast Hypothesis of Lythgoe (1966, 1968). According to the Sensitivity Hypothesis, scotopic visual pigments have evolved to be most sensitive to the light available in the fishes' habitats (they match the photic environment). This hypothesis is most widely accepted for deep-sea fishes, which live in

uniformly blue photic environments and have visual pigments with λ_{max}'s in the blue (Denton and Warren, 1956, 1957; Munz, 1957b, 1958a; Wald et al., 1957). Munz (1958b, 1964) has also presented evidence for a correlation between λ_{max} and the photic environment for epipelagic, rocky-shore, and inshore marine species from the Pacific coast of North America. According to the Contrast Hypothesis, scotopic visual pigments have evolved to maximize visual contrast, and this is achieved if the λ_{max} of the pigment is offset somewhat from the dominant color of the background water. For example, sunlight or skylight reflecting directly from a white or silvery object to the eye is spectrally broader than the background light. Thus an offset visual pigment would give greater contrast between this bright object and the background. Lythgoe developed this hypothesis when he failed to detect a direct relationship between visual pigment absorbance maxima and the photic environment for fishes in the Mediterranean.

In a study of a wide variety of tropical marine fishes, in which the λ_{max}'s of the rhodopsins were correlated with the spectral distribution of the light measured in the animals' habitats during twilight and information on visual behavior, Munz and McFarland (1973) demonstrated convincingly that scotopic visual pigments match the photic environment. Furthermore, since objects are usually seen as dark figures against a lighted background during twilight, the best contrast is given by a pigment maximally sensitive to the background light. Lythgoe had assumed, incorrectly, that light measurements made during the day could be applied to scotopic vision, with appropriate scaling of intensity downwards. (As an example of how slowly the field sometimes progresses,

consider that the study by Munz and McFarland (1973) gave the first good evidence explaining the long- and well-known Purkinje shift!) Maximization of visual contrast by offsetting was probably important in the evolution of photopic (cone) vision in those fishes which have color vision (McFarland and Munz, 1975).

Among the freshwater fishes, most of which have either a porphyropsin alone or a mixed pair of pigments (Schwanzara, 1967), interpretations of function are more speculative because of the lack of detailed correlative studies like that of Munz and McFarland (1973). The argument for significance in visual function rests on a large body of good but circumstantial evidence (see Munz, 1965, and Bridges, 1972). The photic environment in fresh waters tends to be more green, yellow, or red than marine waters; and furthermore, these environments are more variable seasonally or over geologic time periods (Munz, 1965). Thus it would be advantageous to have a visual pigment whose sensitivity is shifted toward the red, as porphyropsins are, and it might be especially advantageous to have changeable proportions of two pigments (ibid.). Those freshwater fishes with changeable proportions tend to have more porphyropsin in the winter months, when the water tends to be more turbid and stained (Allen et al., 1973; only northern temperate fishes have been studied). This hypothesis has been extended to the diadromous fishes, such as the anadromous Pacific salmon, in which there is a succession from rhodopsin to porphyropsin during the spawning migration (Beatty, 1966). An especially interesting case is the catadromous eel Anguilla during its spawning migration, in which the visual pigments change from a "freshwater porphyropsin" to an "epipelagic marine

rhodopsin" by changing chromophores and then to a "deep-sea rhodopsin" by changing opsins (A. anguilla--Carlisle and Denton, 1959; Brown and Brown, quoted by Wald, 1960b; but most convincingly shown in A. rostrata by Beatty, 1975; both species spawn in deep marine waters).

Some workers have remained unconvinced by these arguments (e.g., Lythgoe, 1972a), especially since the photosensitivity of porphyropsins is only 70% that of rhodopsins, when compared at λ_{max} (Dartnall, 1968). However, the photosensitivity of a porphryopsin is much greater than that of the corresponding (same opsin) rhodopsin in the yellow-to-red part of the spectrum, where the majority of available light may be in certain fresh waters, especially during the winter months (Munz, 1965). (The absorbance spectra of porphyropsins are somewhat broader than those of rhodopsins, as well as being shifted toward the red; Munz and Schwanzara, 1967; Bridges, 1967). A full test of the correctness of the Sensitivity Hypothesis in freshwater and diadromous fishes will be difficult. Not only must light measurements be made in the fishes' habitats during twilight and night and information on visual behavior obtained, but day-to-day changes in these variable habitats must be considered. In addition, information on absorption of light in the pre-retinal media and the optical density of the pigments must be obtained, to calculate the percentage of incident photons absorbed by the visual pigments. Let us hope the solution will not be so slow in coming as it was for the Purkinje effect!

For this dissertation I have assumed that the changes in proportions of rhodopsin and porphyropsin in paired-segment species are meaningful. The purposes of this investigation were the following:

1) to gain information on the control of rhodopsin-porphyropsin ratios in rainbow trout (Salmo gairdneri), one race of which is anadromous (steelhead). Effects of temperature, light intensity, and two hormones (prolactin and thyroxine) were studied (Chapters II, III, and IV);

2) to determine whether anadromous sticklebacks (Gasterosteus aculeatus) undergo a succession of visual pigments during the spawning migration, similar to that in anadromous salmonids (Chapter V); and

3) to gain information on the control of visual pigment ratios in sticklebacks, should they be changeable (Chapter VI).

Current information on the control of rhodopsin-porphyropsin proportions is detailed in the following chapters.

CHAPTER II

EFFECTS OF PROLACTIN AND THYROXINE ON THE VISUAL PIGMENTS OF TROUT, *SALMO GAIRDNERI*

The rods of many freshwater and euryhaline teleosts contain both a rhodopsin and a porphyropsin, the relative proportions of which change according to a seasonal or a migratory pattern in some species (reviewed by Bridges, 1972). Administration of thyroxine, whether by injection or by dissolution in the tank water, causes an increase in the percentage of porphyropsin in the salmonids *Salmo gairdneri* (Munz and Swanson, 1965; Jacquest and Beatty, 1972), *Oncorhynchus nerka*, and *O. kisutch* (Beatty, 1972) and in the cyprinid *Richardsonius balteatus* (Allen, 1971). In contrast, Naito and Wilt (1962) have reported that thyroid hormone inhibits the conversion of vitamin A_1 to vitamin A_2 aldehyde (vitamin A_1 aldehyde and vitamin A_2 aldehyde are the prosthetic groups of rhodopsin and porphyropsin, respectively) in eyes of a centrarchid (*Lepomis* sp.) studied in organ culture. However, the retina of this fish contains only porphyropsin at all stages of its life cycle.

In amphibians thyroxine has the opposite effect on the visual pigments. In *Rana catesbeiana* the changeover from pure porphyropsin in the larva to a predominance of rhodopsin in the adult is mediated by thyroxine (Wilt, 1959a,b). Thyroxine is presumed to have a similar effect in *R. temporaria*, *R. pipiens*, and *Hyla regilla*, in which there is a transition from a pure or predominantly porphyropsin retina to a pure rhodopsin retina during metamorphosis (Muntz and Reuter, 1966; Liebman

and Entine, 1968; Crescitelli, 1958). A similar change from vitamin A_2- to vitamin A_1-based pigments occurs in the cones of Rana esculenta and R. pipiens (Muntz and Reuter, 1966; Liebman and Entine, 1968). In some species there is no visual pigment transition during metamorphosis (see Bridges, 1972), but in no known case is there an increase in porphyropsin percentage during thyroxine-induced metamorphosis. Since prolactin has an effect antagonistic to thyroxine in amphibian metamorphosis (see review by Frye et al., 1972) and since a rhodopsin-to-porphyropsin transition associated with the prolactin-induced second metamorphosis has been reported (but never fully documented) in Notophthalmus (=Diemictylus) viridescens (Wald, 1946b, 1947), it has been suggested that prolactin may have an effect on visual pigment conversions in this class (Bern and Nicoll, 1969; Bridges, 1970); but this hypothesis has not been tested. (Note: after this chapter was written, Crim (1975) reported that injections of ovine prolactin increased the percentage of vitamin A_2 in the eyes of Notophthalmus. He also claimed to support Wald's reports that adults have higher percentages of ocular vitamin A_2 than do efts, but his adults were from a permanently aquatic population--i.e., animals which had not gone through the terrestrial eft stage. Since Wald's reports were both abstracts, never published in detail, we still have no good evidence for a natural visual pigment transition in the wild.)

In teleosts prolactin is involved in freshwater hydromineral regulation, especially in euryhaline species. Prolactin is apparently released into the circulation during the migrations of Gasterosteus aculeatus, Anguilla anguilla, and Oncorhynchus nerka into fresh water

(see Lam, 1972, and McKeown and van Overbeeke, 1972). Since a visual pigment change is involved in this migration in several of the species which have been studied (see Bridges, 1972), prolactin may have a visual as well as an osmoregulatory effect in these fishes.[1] The present study was undertaken to determine whether prolactin has an effect on the scotopic visual system of the rainbow trout, Salmo gairdneri.

Materials and Methods

1. Fish

This report is based on two experiments, the first performed during July, 1972, and the second during December, 1972, and January, 1973. The rainbow trout, Salmo gairdneri, was used in both experiments. The average size of the fish was 75 grams in the first experiment and 100 grams in the second. They were sexually immature and of both sexes.

2. Experimental Conditions

The fish were kept in an experimental coldroom maintained at 12 $\pm$ 1° C. They were divided into 4 groups, each group in a separate 64-liter fiberglass tub coated with white tygon paint. Each tub was illuminated from above by three 150-watt blue dichroic spotlights (G.E.) shining through a blue plexiglass filter. The spectral distribution of this light source was illustrated by Allen (1971, Fig. 1). Intensity was controlled by regulating the voltage to the lamps; it was adjusted to the same level in each tub by measuring the spectral intensity (at

[1] Gasterosteus has both rod pigments (Munz, 1957a), but changes in their proportions have not been studied.

475 nm) near the water surface with an Instrumentation Specialties Company (ISCO) SR spectroradiometer. The light:dark cycle was 15:9 (Experiment 1) or 9:15 (Experiment 2), each a natural photoperiod for the time of year. The lights were turned on and off gradually over a 1 minute interval with dimmer switches to avoid startling the fish.

The fish were allowed either 2 days (Experiment 1) or 12 days (Experiment 2) to adapt to these conditions before hormonal treatment was started. A fifth group was formed by taking an equal number of animals from each tub either immediately (Experiment 1) or 8 days (Experiment 2) after procurement of the fish. This group, called the initial batch, was then dark-adapted and sacrificed for analysis of its visual pigments.

Fish were fed fresh chopped earthworms.

3. Hormonal Treatment

Ovine prolactin (NIH-P-S10, 25.6 IU/mg) was dissolved in a solution of 0.7% NaCl and 0.53% Na_2HPO_4, pH adjusted to 7.7 (Experiment 1) or in 0.6% NaCl and 0.27% Na_2HPO_4, pH adjusted to 8.3-8.5 (Experiment 2); prolactin concentrations were 0.5 mg/ml and 1.875 mg/ml, respectively. The prolactin was much more soluble in the latter saline.

First Experiment.--Groups 1 and 3 were injected intraperitoneally every third day with 2 μg prolactin per gram body weight; groups 2 and 4 were injected with saline only. Injections were given during the last 6 hours of the light period. L-thyroxine sodium pentahydrate (Nutritional Biochemicals) was dissolved in the tank water of groups 3 and 4 to a concentration of 1 mg/liter. The water in each tank was replaced every

third day (on the day of injections) with dechlorinated tap water. The fish in groups 1 and 2 were sacrificed on the tenth day of the experiment (injections given on days 1, 4, and 7 but not 10). This half of experiment 1 was terminated earlier than planned due to death of fish bringing the sample size close to estimated minimum number necessary for meaningful statistical analysis. Groups 3 and 4 were sacrificed on day 18, 56-60 hours after the sixth injection.

Second Experiment.--The treatment was similar except that the prolactin dose was 15 μg/g every other day and the throxine concentration was 10 μg/liter. Injections were given within hours 2½-7 of the light period. Approximately half of the trout in each group were sacrificed 32-36 hours after the ninth injection; the remaining, 32-36 hours after the tenth injection. This arrangement was necessary because of the large number of retinae to be processed; there were no casualties during this experiment. Tank water was changed every other day, on the day of injections. A mixture of salts was also added to each tank in this experiment; concentrations of added salts were 0.09 mM NaCl, 0.01 mM KCl, 0.08 mM $MgSO_4$, and 0.30 mM $CaCO_3$.

The following mnemonic codes for the 5 groups in each experiment will be used: I (initial batch), Pl (prolactin-injected, no thyroxine in water), S (saline-injected, no thyroxine in water), T_4-Pl (prolactin-injected, thyroxine in water), and T_4-S (saline-injected, thyroxine in water).

4. Analysis of Visual Pigments

Fish were dark-adapted at least 5 hours before the retinae were removed. The 2 retinae of each fish were pooled to make one sample. Visual pigment extracts were prepared and later examined with a Cary Model 14 spectrophotometer using the method of partial bleaching as described in Munz and Beatty (1965). A computer program calculated the difference spectra and the percentages of $VP503_1$ and $VP527_2$ therefrom (Munz and Allen, 1968). The notation $VP\lambda_n$ indicates a visual pigment based on vitamin A_n, where n=1 or n=2, and whose maximum absorbance occurs at λ nanometers. Hence $VP503_1$ is trout rhodopsin and $VP527_2$ is trout porphyropsin. The absorbance percentages were converted to molar percentages using the values 40,600 and 30,000 for the molar extinctions at λ_{max} of $VP503_1$ and $VP527_2$, respectively (Wald and Brown, 1953; Brown et al., 1963; Dartnall, 1968).

5. Statistical Tests

The molar percentage of $VP527_2$ was arbitrarily chosen as the variable for comparisons and presentation of data. When the pigment percentage is near 0 or 100%, the distribution becomes skewed away from the extreme, and the variance is smaller. Allen (1970) found empirically that the arcsine transformation corrects for this non-normality. (This observation is not a consequence of the binomial distribution, because the number of molecules on which each percentage is based is quite large.) Consequently the statistical analysis was performed on the arcsine-transformed data. Means and their 95% confidence limits were calculated using the angular scale, but they are

reported as percentages of $VP527_2$.

Statistical comparisons were made using a model I analysis of variance (all of the procedures below which are not referenced may be found in Sokal and Rohlf, 1969). The following sets of comparisons was planned *a priori* in each experiment: μ_I vs. μ_S ; μ_{Pl} vs. μ_S ; $\mu_{T_4\text{-}Pl}$ vs. $\mu_{T_4\text{-}S}$; and $\frac{1}{2}(\mu_{Pl} + \mu_S)$ vs. $\frac{1}{2}(\mu_{T_4\text{-}Pl} + \mu_{T_4\text{-}S})$, where μ_X is the mean of the population from which group X is sampled.[2] The F_{max}-test and Bartlett's test were used to test for homogeneity of variances. For the second experiment Bartlett's test was significant at the 5% level, and the F_{max}-test was within rounding error of the borderline between non-significance and the 5% level. This mild heteroscedasticity was judged not to affect the analysis seriously. For the first experiment, however, both tests were highly significant. The variances of the two thyroxine-treated groups were extremely low compared with those of the other three groups (see Figure 2.1). Therefore, a modification of a procedure suggested by Snedecor and Cochran (1967, p. 324) was employed. A separate MS_w (mean square within groups) was calculated for I, Pl, and S and for T_4-Pl and T_4-S. The *a priori* comparsions μ_I vs. μ_S, μ_{Pl} vs. μ_S, and $\mu_{T_4\text{-}Pl}$ vs. $\mu_{T_4\text{-}S}$ were

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[2] If μ_I were compared with the average of the other four means instead of with μ_S, the set would be mutually orthogonal. However, since that comparison is of little biological interest, the advice of Sokal and Rohlf (1969, p. 463) has been followed. In these two experiments, if the comparisons μ_I vs. μ_S had been made with the *a posteriori* test discussed in the next paragraph, the significance levels would have been no different from those to be reported.

made using the appropriate MS_w and degrees of freedom. The comparison $\frac{1}{2}(\mu_{Pl} + \mu_S)$ vs. $\frac{1}{2}(\mu_{T_4\text{-}Pl} + \mu_{T_4\text{-}S})$ was made with a special t-test for non-homogeneous variances (ibid.).

All comparisons suggested by the data (a posteriori) were made with Gabriel's sum of squares simultaneous test procedure. In the first experiment the MS_w for this test was chosen as described above.

Results

Experiment 1

Results of the first experiment are shown in Table 2.1 and Figure 2.1. Initially the retinae had a high porphyropsin content (group I, 76.8%), which decreased significantly ($p < .001$) in the saline-injected controls (S, 46.1%). Factors responsible for this shift may include some aspect of the lighting (Dartnall et al., 1961; Bridges, 1964a, 1965a; Allen, 1971), the temperature (Allen and McFarland, 1973), or the fact that these fish had been fed with commercial trout food, which contains vitamin A_2, possibly causing these fish to have an artificially high proportion of porphyropsin (see Beatty, 1972). Their diet during the experiment (earthworms) contained only vitamin A_1-precursors, as in their natural diet.

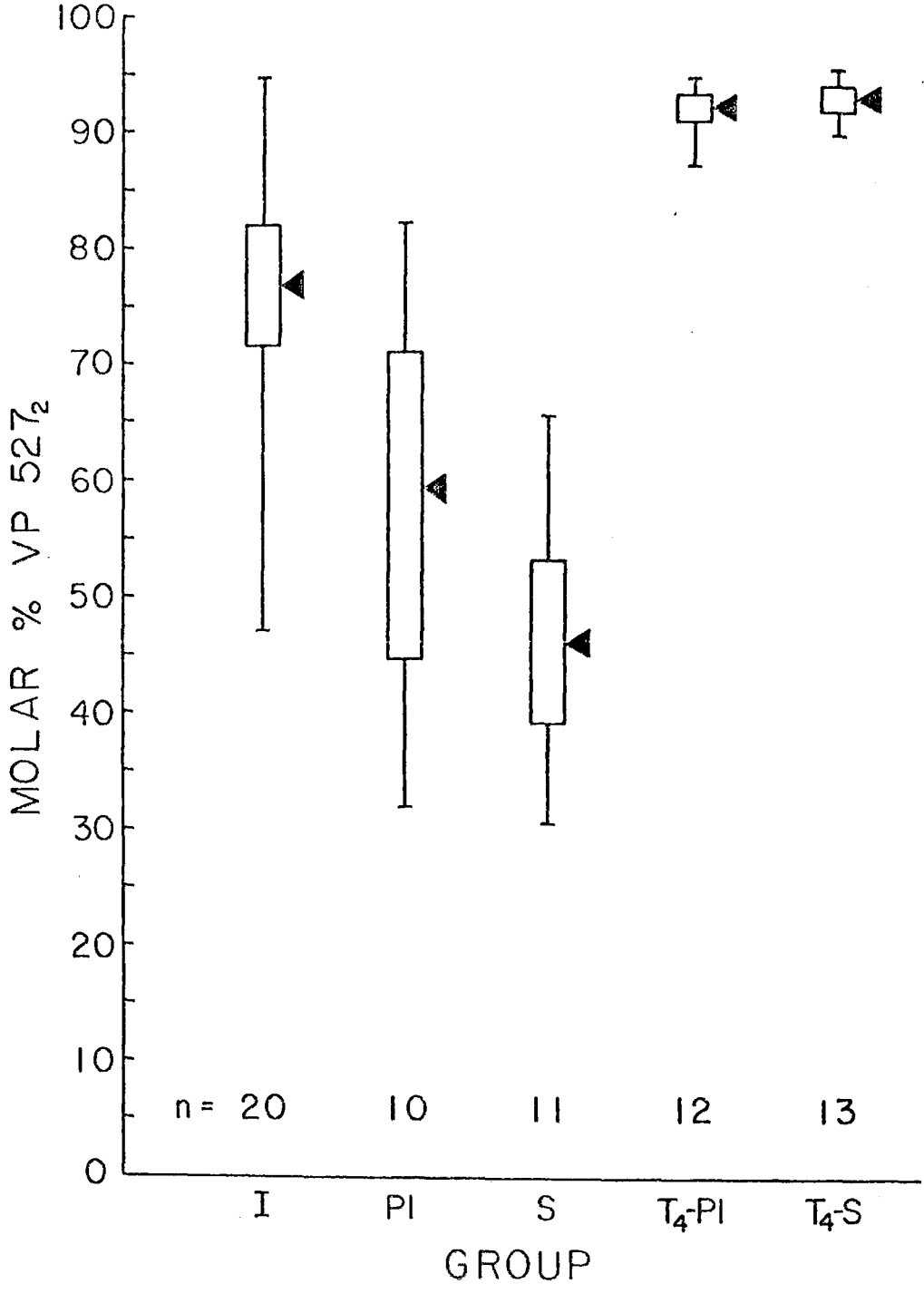
The prolactin-injected group also showed a significant decrease ($p < .01$) from the initial batch (Pl, 59.4%) when tested a posteriori, but the percentage of $VP527_2$ was greater in this group than its control at a low significance level ($p < .05$).

When thyroxine was added to the water at a concentration of 1 mg/l, there was no significant difference between the prolactin- and

Table 2.1.--Mean molar percentage of $VP527_2$, its 95% confidence limits, and number of animals in each group of each experiment

Experiment number	Group mnemonic	Number of animals	Mean % $VP527_2$	95% C.L. of mean	Description of group
1	I	20	76.8	71.5-81.8	initial batch
	P1	10	59.4	47.2-71.0	prolactin-injected, 2 μg/g every third day, 3 injections
	S	11	46.1	39.1-53.2	saline-injected control
	T_4-P1	12	91.9	90.7-93.0	prolactin-injected, 2 μg/g every third day, 6 injections; and 1 mg/l thyroxine in water
	T_4-S	13	92.6	91.5-93.7	saline-injected control with 1 mg/l thyroxine in water
2	I	8	80.4	75.8-84.5	initial batch
	P1	20	88.5	86.8-90.0	prolactin-injected, 15 μg/g every other day, 9 or 10 injections
	S	17	53.9	48.2-59.6	saline-injected control
	T_4-P1	16	87.4	84.8-89.8	prolactin-injected, 15 μg/g every other day, 9 or 10 injections; and 10 μg/l thyroxine in water
	T4-S	16	66.4	60.6-71.9	saline-injected control with 10 μg/l thyroxine in water

Figure 2.1.--Experiment 1: Mean molar percentages of $VP527_2$ (arrows) and their 95% confidence limits (bars). Lines represent the range. The number of animals in each group (n) is given above the group mnemonic. Groups: I, initial batch; Pl, prolactin-injected, 2 μg/g every third day, 3 injections; S, saline-injected control; T_4-Pl, prolactin-injected, 2 μg/g every third day, 6 injections, and 1 mg/l thyroxine in water; T_4-S, saline-injected control with 1 mg/l thyroxine in water.


100
90
80
70
60
50
40
30
20
10
0
MOLAR % VP 527₂
n = 20
10
11
12
13
I
PI
S
T₄-PI
T₄-S
GROUP

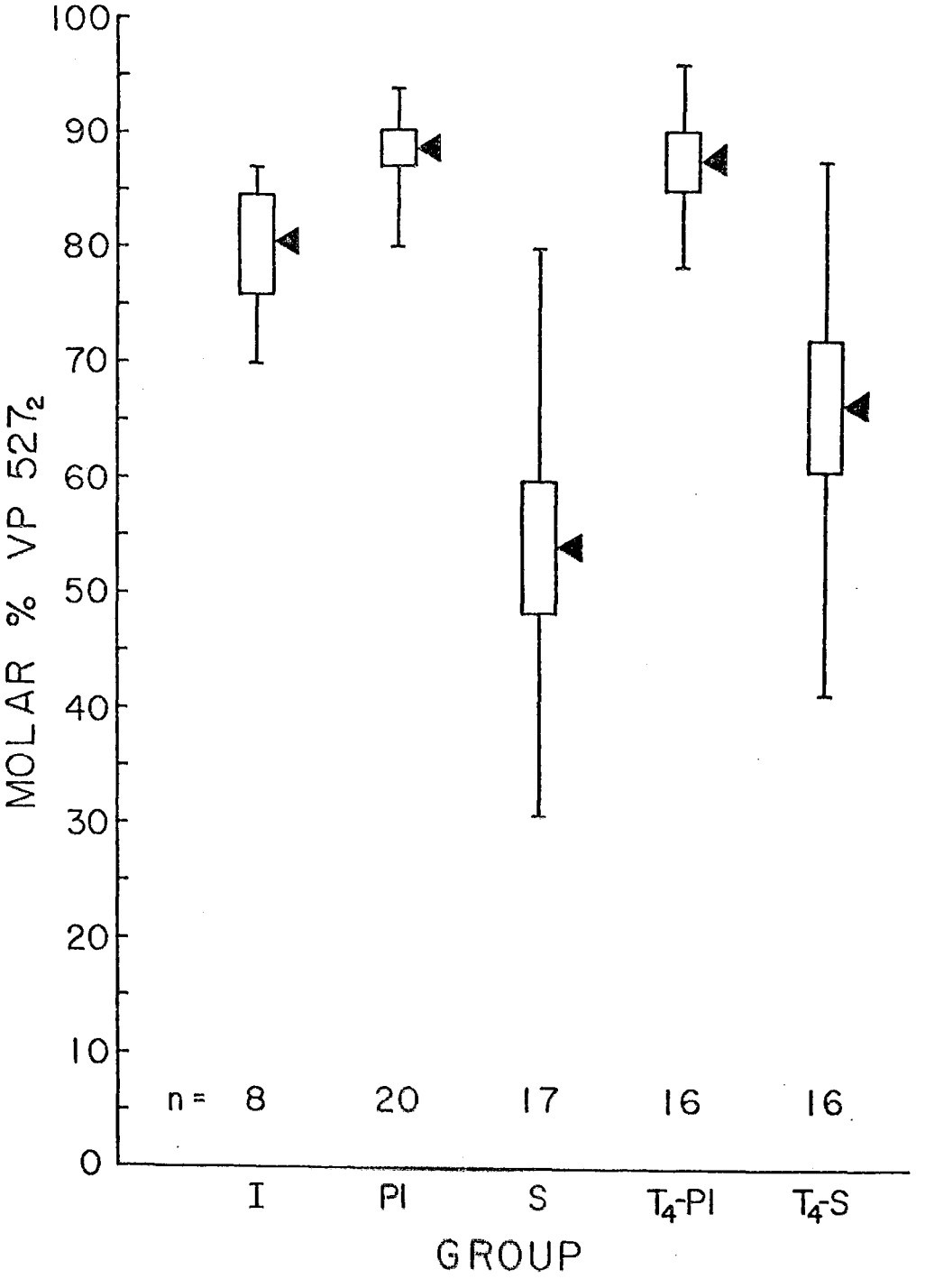
saline-injected groups (T_4-Pl, 91.9%; and T_4-S, 92.6%). However, the concentration of thyroxine used has apparently forced the proportion of porphyropsin near its upper limit, masking possible effects of prolactin. Note the decrease in variance, which is highly significant ($p < .001$). The difference between the average of the means of the two thyroxine groups and the average of the means of the corresponding two groups without thyroxine is also highly significant ($p < .001$).

Experiment 2

There was no significant difference between the half of each group receiving nine injections and the half receiving ten (t-test). Therefore, no distinction is drawn between them in analyzing the results, shown in Table 2.1 and Figure 2.2. Here again the saline-injected control group (S) has a significantly ($p < .001$) lower percentage of porphyropsin than the initial batch (53.9% vs. 80.4%), even though the initial batch was kept under the experimental conditions for eight days before it was sacrificed. Four tagged fish which were kept with the saline-injected control group but were not injected had a mean porphyropsin percentage of 58.7 (95% C.L. of 47.3-69.6), significantly different from the initial batch ($p < .001$) but not the control group (t-tests). Hence it is unlikely that the saline injections had a role in this shift.

In this experiment, in which the prolactin injections were more numerous, more frequent, and of higher dosage, the prolactin-injected group (Pl) had a highly significantly ($p < .001$) greater percentage of $VP527_2$ than its control (S) group (88.5% vs. 53.9%). There was also a

Figure 2.2.--Experiment 2: Mean molar percentages of $VP527_2$ (arrows) and their 95% confidence limits (bars). Lines represent the range. The number of animals in each group (n) is given above the group mnemonic. Groups: I, initial batch; P1, prolactin-injected, 15 μg/g every other day, 9 or 10 injections; S, saline-injected control; T_4-P1, prolactin-injected, 15 μg/g every other day, 9 or 10 injections, and 10 μg/l thyroxine in water; T_4-S, saline-injected control with 10 μg/l thyroxine in water.


100
90
80
70
60
50
40
30
20
10
0
MOLAR % VP 527₂
n = 8 20 17 16 16
I
PI
S
T₄-PI
T₄-S
GROUP

highly significant difference between the prolactin-injected group and its control when 10 μg/l thyroxine was added to the tank water, the former group again having more porphyropsin (T_4-Pl, 87.4% vs. T_4-S, 66.4%).

Does thyroxine at this lower dosage have an effect? Comparison of the average of the two thyroxine groups with the average of the two corresponding non-thyroxine groups yielded no significant difference, but an a posteriori comparison of S (53.9%) vs. T_4-S (66.4%) did show a significant difference ($p < .005$). There was no significant difference between the two prolactin groups (88.5% vs. 87.4%).

Discussion

It is quite clear that both prolactin and thyroxine at their higher dosages favor porphyropsin in this fish. At the lower dosages used in this study, the effects of either hormone are somewhat equivocal, since the differences between the hormone-treated group and its control are significant when the other hormone is absent and are non-significant when it is present at the high dosage. In the former case, however, the effect is in the same direction as at higher dosages (hormone favors VP 527_2), and in the latter case the percentages of $VP527_2$ of both groups are high, perhaps decreasing the likelihood of seeing a small effect.

The thyroxine effects are grossly similar in magnitude to those seen in Richardsonius by Allen (1971). He dissolved thyroxine in the tank water at concentrations of ten, 100, and 1000 μg/liter and measured the percentage of porphyropsin in the fish after ten days. With

the lowest dosage there was a small but significant increase in porphyropsin compared with controls (24.8% vs. 12.1%), and with the higher two doses there was a large increase (62.3% and 72.5% for the medium and high doses, respectively, vs. 12.1% for the controls). Thyroxine dosages in my experiments were 1000 μg/liter for 17 days (Experiment 1) and 10 μg/liter for 17 or 19 days (Experiment 2) (and compare Figures 2.1 and 2.2).

Effects of prolactin on visual pigments have not been previously reported, but the higher dose (15 μg/g alternate days) is similar to those used in fish osmoregulation studies (Ball, 1969). One must be cautious in drawing conclusions, however, for mammalian prolactin stimulates the thyroid, apparently via TSH secretion, in the eel Anguilla (Olivereau, 1968) and the cichlid Pterophyllum scalare (Osewold and Fiedler, 1968) but not in Poecilia latipinna (Higgins and Ball, 1972) or Gasterosteus (Leatherland and Lam, 1971, and their unpublished results quoted by Lam, 1972) and perhaps not in the cichlid Cichlasoma biocellatum (Mattheij et al., 1972). The trout in this study were not hypophysectomized.

If the effect is genuine, does it fit into the natural history of the fish? Unfortunately both the visual pigment and the hormonal studies on migrating fish are spotty and not without interpretational difficulties. A brief summary will be attempted nevertheless. During or just before the upstream migrations of the lamprey Petromyzon marinus (Wald, 1957) and three species of the Pacific salmon, Oncorhynchus (Beatty, 1966), there is a change from rhodopsin- to porphyropsin-dominated retinae (for a discussion of the possible visual

advantages of this transition, see Munz (1965); the argument rests on the fact that the ocean light environment is bluer than that of fresh waters). The pigments of the eel Anguilla have not been studied during this migration, but the reverse transition (porphyropsin to rhodopsin) occurs during the seaward migration (Carlisle and Denton, 1959; Brown and Brown, quoted by Wald, 1960b; shown most convincingly by Beatty, 1975). A similar changeover must occur in salmonids: juveniles in fresh water have varying proportions of the two pigments, whereas ocean-caught fish have mostly rhodopsin (Beatty, 1966). The timing of this transition is not known. Histological studies indicate that a discharge of prolactin is associated with the upstream migrations of Anguilla (Vollrath, 1966) and Gasterosteus[3] (Leatherland, 1970a). Serum levels of prolactin have been measured in the sockeye salmon, Oncorhynchus nerka, during its spawning migration up the Fraser River (McKeown and van Overbeeke, 1972). Compared with seawater fish, there was an increase of about 55% in serum prolactin as the fish neared the mouth of the river, but there was a decrease of 27% in fish caught 30 miles upstream from the mouth. Fish taken 200 and 375 miles from the mouth, the latter at the spawning site, again showed increased levels of serum prolactin compared with seawater fish (75 and 81%, respectively). Although the dip in prolactin concentration as the fish enter fresh water, which they confirmed in an experiment the following year, is difficult to interpret, there seems to be an overall increase during the migration. Increased thyroid activity is associated with the

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[3]See footnote 1.

ocean-to-fresh water migrations of Anguilla (see D'Ancona, 1960), Gasterosteus (Baggerman, 1957, 1962; Hoar, 1965; Leatherland, 1970b), and the Atlantic salmon, Salmo salar (Leloup and Fontaine, 1960). Thus the hypothesis that both thyroxine and prolactin are involved in the rhodopsin-to-prophyropsin conversion is attractive. The time of action of these two hormones may not be completely simultaneous, for the thyroid probably becomes less active at the end of the migration (Leloup and Fontaine, 1960; Leatherland, 1970b), whereas the prolactin levels presumably remain high because of its role in freshwater osmoregulation. Perhaps prolactin also serves to maintain the high porphyropsin levels --Allen (1971) found a partial reversal of the thyroxine effect in Richardsonius after hormone treatment was curtailed. A problem presents itself immediately, however. Increased thyroid activity is also associated with the seaward migration of Anguilla (see d'Ancona, 1960) and the parr-to-smolt metamorphosis in salmonids (see Hoar, 1965).

It is interesting that the prolactin effect is the same as that postulated for amphibians, but in light of the opposite effects of thyroxine on teleost and amphibian visual pigments, further comparative studies of both thyroxine and prolactin effects are necessary. A study on the lamprey (a cyclostome) might also be rewarding.

CHAPTER III

EFFECTS OF TEMPERATURE AND LIGHT INTENSITY ON THE VISUAL PIGMENTS OF RAINBOW TROUT

Large changes in the control groups under laboratory conditions has impeded the study of effects of hormones, light, or other parameters on rhodopsin-porphyropsin ratios in fishes (e.g., Cristy, 1974; Allen, 1971). In a study of the effects of prolactin and thyroxine in rainbow trout, the control groups decreased substantially from initially high percentages of porphyropsin, whereas the hormone-treated groups increased slightly from the initial groups or decreased to a lesser extent than the corresponding control groups (Cristy, 1974). Although the means of the hormone-treated groups were significantly higher than the means of their respective control groups, this lack of control of the "control" groups made interpretation of the results more difficult. The experiments reported here were designed to gain information on how to manipulate the rhodopsin-porphyropsin ratio in trout to a desired level, and emphasis was placed on holding the visual pigments at low and constant percentages of porphyropsin for hormonal studies. These experiments achieve that goal; they also give further insight into the combined effects of temperature and light intensity in setting pigment ratios.

The rhodopsin-porphyropsin ratio in many fishes changes seasonally, the proportion of porphyropsin being higher in winter than in summer (see Allen et al., 1973). From experiments with a cyprinid,

the rudd (Scardinius erythrophthalmus), in which light favored rhodopsin and darkness favored porphyropsin, seasonal changes in light (intensity and/or photoperiod) were believed to be responsible for the seasonal changes in visual pigments (Dartnall et al., 1961). A poeciliid, Belonesox belizanus, and a second cyprinid, Notemigonus crysoleucas, were later shown to respond to light and darkness in the same way (Bridges, 1965a; Allen and McFarland, 1973).

The situation has become more complex recently with the finding that a third cyprinid, Richardsonius balteatus, responds in the opposite fashion to experimental light and darkness, although the seasonal changes are similar to those in the rudd (Allen, 1971). A similar opposite response occurs in rainbow and brook trout (Salmo gairdneri and Salvelinus fontinalis); and cutthroat trout (Salmo clarki) from bright, open areas of a mountain stream have higher proportions of porphyropsin than do fish from adjacent forested areas, which are less brightly illuminated (Allen et al., 1973). Hence fishes may be classified as "rudd responders" or "anti-rudd responders," according to the effects of light and darkness on their visual pigments.

Allen et al. (1973) have proposed that seasonal fluctuations in temperature may be responsible for the seasonal shifts in visual pigment proportions, at least in "anti-rudd responders." Evidence to support this hypothesis has been reported for rainbow trout (an "anti-rudd"), Notemigonus (a "rudd"), and the cyprinid Notropis cornutus (McFarland and Allen, 1973; Allen and McFarland, 1973).

Earlier workers had reported that light or brightness favors rhodopsin in several salmonids (juvenile Pacific salmon, Beatty, 1966;

rainbow and brown trout, Dartnall, 1962), contrary to the above findings in rainbow, cutthroat, and brook trout by Allen et al. (1973). The light intensity effects reported here in juvenile rainbow trout (steelhead race) are an "anti-rudd" type, and temperature effects support the hypothesis of Allen et al. (1973), outlined in the previous paragraph.

Materials and Methods

1. Fish

Juvenile rainbow trout (Salmo gairdneri, steelhead race), average weight 29 grams (range 8-59 grams), were obtained from the Leaburg Trout Hatchery, Leaburg, Oregon, on March 3, 1975. The water temperature at the hatchery was 5° C.

2. Experimental Conditions

The fish were brought immediately into an experimental coldroom to test effects of temperature, light intensity, and artificial diets. They were divided into 7 groups, each group into a separate 64-liter fiberglass tub coated with white Tygon paint.

Each tub was illuminated by three 150-watt blue dichroic spotlights (G.E.), 80 cm above the water surface, shining through a blue plexiglass filter (#2424, 0.125 inches thick). Intensity was controlled by regulating the voltage to the lamps; at the highest intensity (group I3, Table 3.1), the lamps were at full line voltage (115V). Irradiance near the water surface was measured with a Gamma 3000R scanning spectroradiometer, which reads directly in photons$\cdot cm^{-2} \cdot nm^{-1} \cdot sec^{-1}$. A computer program, written by D. M. Allen, calculates the total number of

photons·cm^{-2}·sec^{-1} between 410 and 700 nm and the wavelengths at which the 25%, 50%, and 75% points of the cumulative photon frequency distribution occur (the λP_{25}, λP_{50}, λP_{75} points; Munz and McFarland, 1973). For the light source described above, the λP_{25}, λP_{50}, and λP_{75} values are 466, 483, and 498 nm, respectively; the spectral distribution, in energy units, was illustrated by Allen (1971; Fig. 1; blue light). Since the light intensity at the surface was not uniform, an average value was determined from measuring the peak irradiance at 16 points. The light intensities for the various groups are listed in Table 3.1. The light:dark cycle was 11½:12½, close to the photoperiod when the fish were obtained.

Fish in the most dimly illuminated tanks were maintained at one of four temperatures--2.5°, 7°, 12°, or 16° C (see Table 3.1). All other groups were kept at 12° C. The temperatures of the two colder groups were controlled by circulating cold water, the temperature of which was controlled by Aminco and Lauda laboratory baths, through thinly painted copper coils in the trout tubs; the temperature of the 16° group was controlled with a heater tape-thermostat combination; and the temperatures of the 12° groups were controlled by adjusting the room temperature and the aeration in the tubs. Tubs were brought gradually to the appropriate temperature within 24 hours (2.5° and 7°) or 48 hours (12° and 16°).

Water was static but constantly filtered with small charcoal box filters and aerated. A mixture of salts was added to the dechlorinated tap water in each tub (local tap water is low in ions); concentrations of added salts were 1.5 mM $CaCl_2$, 0.4 mM NaCl, 0.05 mM KCl, and 0.5 mM

Na_2HPO_4. The $CaCl_2$ is important in reducing mortality during the high-stress period after procurement of the fish (unpublished personal observations).

Fish were fed lightly (1 gram dry weight per 400 grams fish per day) with chopped earthworms or with Sterling Silver Cup trout chow (see Table 3.1).

3. Analysis of Visual Pigments

Fish were dark-adapted at least 3 hours before the retinae were removed. The two retinae of each fish were pooled to make one sample. Retinal extracts were prepared as described in Allen et al. (1973), except that 0.5 ml of 2% digitonin and 0.05 ml of saturated sodium borate were used. Spectra were obtained with a Cary Model 14 spectrophotometer; extracts were bleached to completion (6 min) with orange light (610 nm dominant wavelength) and a second set of spectra obtained. A computer program calculated the difference spectra and the percentages of $VP503_1$ (trout rhodopsin) and $VP527_2$ (trout porphyropsin) therefrom (Munz and Allen, 1968). The absorbance percentages were converted to molar percentages using the values 40,600 and 30,000 for the molar extinctions at λ_{max} of $VP503_1$ and $VP527_2$, respectively (Wald and Brown, 1953; Brown et al., 1963; Dartnall, 1968).

4. Statistical Tests

Statistical tests were performed with model I analysis of variance and regression analysis; comparisons suggested by the data (a posteriori) were made with Gabriel's sum of squares simultaneous test procedure

(Sokal and Rohlf, 1969). The arcsin transformation was used to correct for non-homogeneity of variance (Finney, 1964, pp. 75-80).

Results

Artificial Diet

In a previous experiment, trout moved from the hatchery to the lab decreased their porphyropsin content dramatically (Cristy, 1974). Fish at the hatchery were fed commercial trout chow, which contains fish meal and consequently a mixture of vitamins A_1 and A_2. Fish in the lab were fed earthworms, which contain vitamin A_1-precursors only. Although it was postulated that lighting and/or temperature differences were responsible for the shift, the possibility that hatchery fish have artificially high porphyropsin content because of their diet could not be ruled out. (It was considered an unlikely factor here, because trout chow is heavily doped with synthetic vitamin A_1. Diet can affect the visual pigments: Jacquest and Beatty (1972) have shown that trout increase their proportion of porphyropsin when fed with walleye liver, a concentrated source of vitamin A_2.)

Two groups of trout were kept at a temperature and light intensity expected to reduce the percentage of porphyropsin, one fed earthworms and the other fed trout chow (groups T12 and Chow, Table 3.1). Each group, sampled after 2 and 4 weeks, dropped in in %VP527, and they were not significantly different from each other.

Table 3.1. Complete data of experiment.

Group mnemonic[A]	Time (weeks)	Temp. (°C)[B]	Light intensity[E]	Food	Number of animals	Mean molar % $VP527_2$	95% C.L. of mean
Initial	0	5[C]			12	72.4	65.0 - 80.6
T12 or I1	1	11.9 ± 0.5	1.773×10^{13}	earthworms	12	56.4	52.2 - 60.7
T2.5	2	2.5 ± 0.3	1.773×10^{13}	earthworms	12	71.2	66.1 - 76.8
T7		7.0 ± 0.2	"	"	12	60.1	51.8 - 68.9
T12 or I1		11.9 ± 0.5	"	"	12	39.2	31.4 - 46.7
Chow		11.9 ± 0.5	"	trout chow	12	34.8	28.3 - 40.9
T16		16.0 ± 0.1	"	earthworms	12	19.1	14.3 - 23.3
I2		11.9 ± 0.7	5.911×10^{13}	"	11	40.7	34.1 - 47.0
I3		12.0 ± 1.0	3.694×10^{14}	"	12	55.7	47.5 - 64.1
T12 or I1	3	see above	see above	see above	12	21.2	14.5 - 27.1
T2.5	4	footnote D	see above	see above	12	61.3	54.6 - 68.3
T7		see above			12	39.2	34.0 - 44.3
T12 or I1					13	16.8	12.3 - 20.6
Chow					12	12.8	7.1 - 17.3
T16					11	2.5	0.3 - 3.7
I2					11	26.2	16.7 - 34.8
I3					12	46.2	38.9 - 53.4

[A] T for "temperature" and I for "light intensity."

[B] Average temperature ± typical daily variation.

[C] Temperature of water at hatchery.

[D] Temperature rose to 4-5 °C for about 12 hours on day 18 because of failure of regulatory system.

[E] photons·cm^{-2}·sec^{-1} [410-700 nm] ; "blue" light--see Methods; light:dark cycle = 11½:12½ .

Temperature

At the lowest light intensity, fish were kept at each of four temperatures--2.5°, 7°, 12°, and 16° C--and sacrificed at intervals up to 4 weeks (Table 3.1 and Figure 3.1). All groups were decreasing in %VP527, but there was an obvious effect of temperature evident at both two and four weeks (linear regression is significant at $P < .05$ and $P < .01$, respectively).

The important question is: are all the groups tending toward the same steady-state level determined by the light intensity, or are they approaching different levels determined by both temperature and light intensity? For sake of argument let us assume the former--each group is exponentially approaching 0% VP527, but at different rates because metabolism is faster at higher temperatures. With this assumption we can calculate these rate constants and thus Q_{10} values for the temperature intervals 2.5°-7°, 7°-12°, and 12°-16°, and see whether the Q_{10} values are reasonable, as a crude test of the assumption. These values at 18.1, 5.7, and 8.1, respectively. Temperature coefficients (Q_{10}'s) are ordinarily between 2 and 3; in poikilotherms, the presence of isoenzymes or different temperature states of an enzyme may cause the Q_{10} to approach 1 (Hochachka and Somero, 1971). Therefore the latter hypothesis, that different steady-state levels are being approached, is more likely.

Light Intensity

At 12° C trout were kept at three different light intensities (Table 3.1 and Figure 3.2). The %VP527 fell more rapidly in dimmer

Figure 3.1.--Effects of temperature on porphyropsin percentage. X: initial batch; circles: 2.5° C; triangles: 7°; filled squares: 12°; open squares: 12°, but fed trout chow instead of earthworms; hexagons: 16°. Vertical lines represent the 95% confidence limits of the means. All groups were held in dim "blue" light (see Methods and Table 3.1.

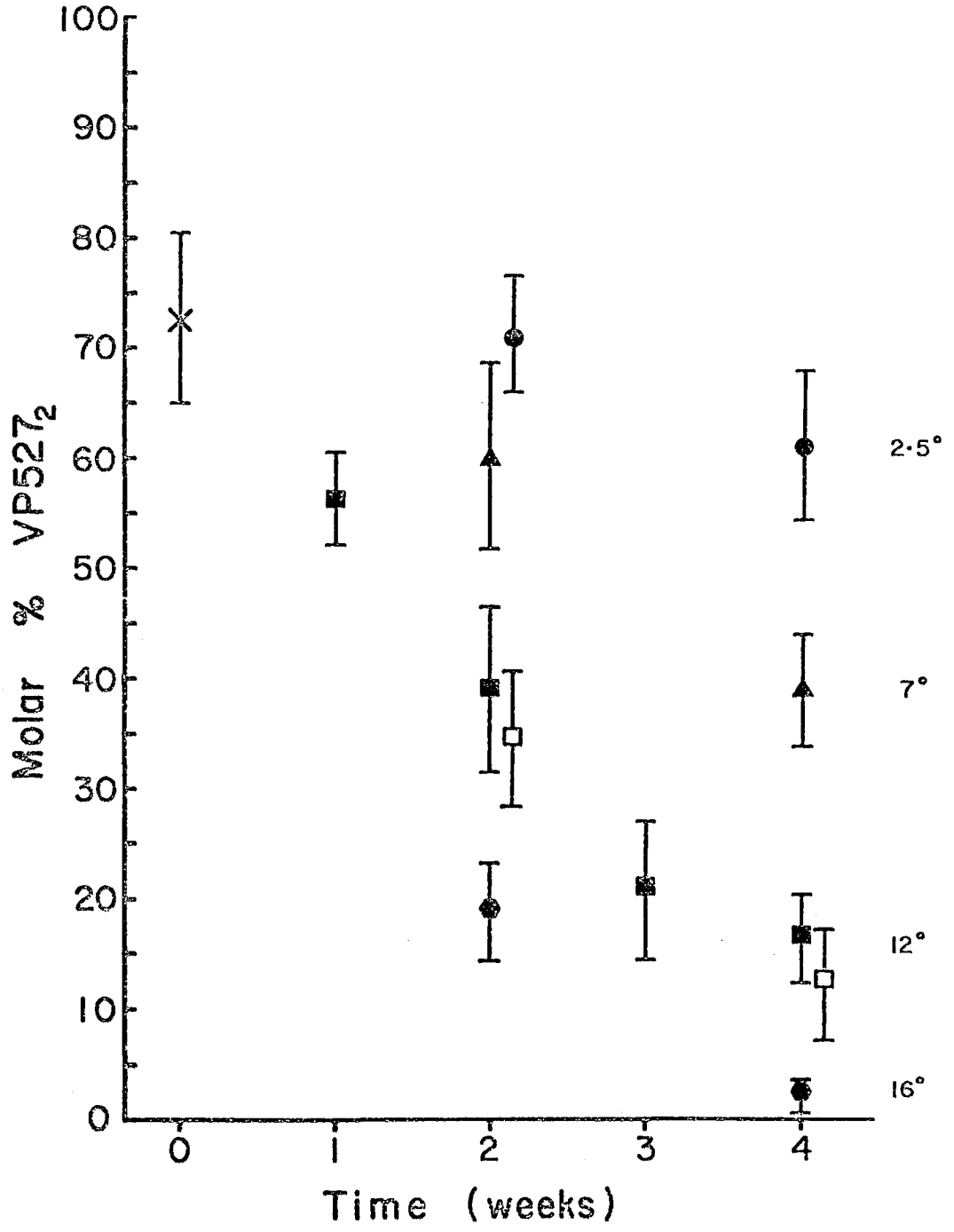
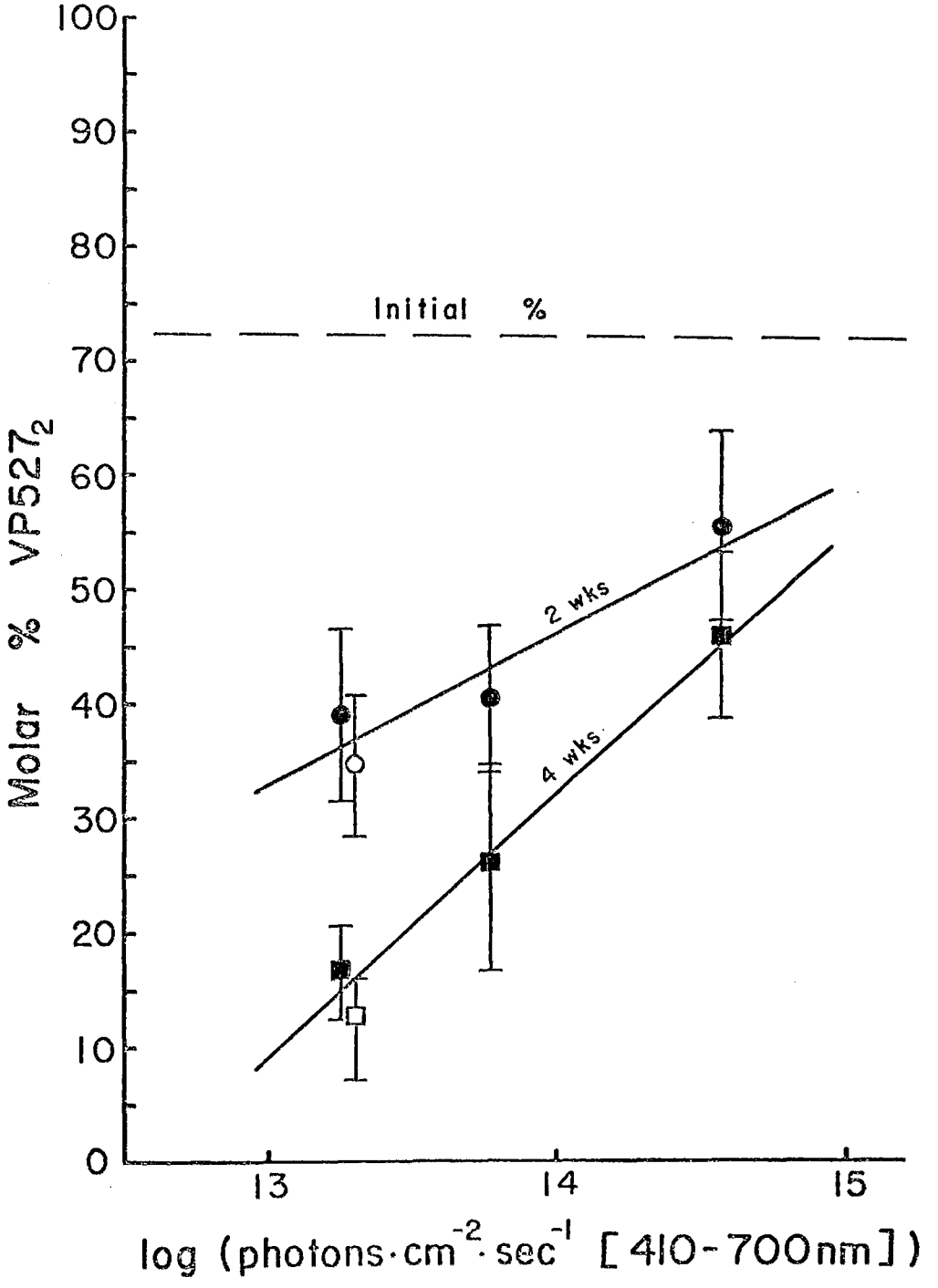

100
90
80
70
60
50
40
30
20
10
0
Molar % VP527$_2$
0
1
2
3
4
Time (weeks)
2·5°
7°
12°
16°

Figure 3.2--Effects of light intensity on porphyropsin percentage. Dashed line represents the %VP527 at start of experiment. Circles: after 2 weeks of treatment; squares: after 4 weeks of treatment; open symbols: fed trout chow instead of earthworms. Vertical lines represent the 95% confidence limits of the means. All groups were held at 12° C in "blue" light (see Methods). The regression analysis was done with the data represented by filled symbols only, using the arcsine transformation (see Results). The straight lines here are eye-fitted to the untransformed data, including the open symbols.


100
90
80
70
60
50
40
30
20
10
0
Initial %
Molar % VP527$_2$
2 wks
4 wks
13
14
15
log (photons·cm^{-2}·sec^{-1} [410-700nm])

light. Linear regression (arcsin $\sqrt{\%VP527 \div 100}$ vs. log intensity) is not significant at 2 weeks, but an a posteriori test revealed that the mean of the highest intensity group (I3) is significantly ($P < .01$) greater than the means of the other two (I1 and I2). Linear regression is significant at 4 weeks ($P < .05$). The group fed trout chow is also shown in Figure 3.2, but it was not figured in the statistical analysis (inclusion would increase the significance levels).

Discussion

The original goal of these experiments, to gain manipulative control over the rhodopsin-porphyropsin system in rainbow trout, has been achieved. An empirical equation to predict the percentage of porphyropsin after four weeks of a given light intensity-temperature treatment has been developed:

$$Y = -4.7T + 24.4\log I - 251 ,$$

where Y is the molar %VP527, T is the temperature in °C, and I is the light intensity in photons$\cdot cm^{-2} \cdot sec^{-1}$ [410-700 nm]. This equation applies only for a "blue" light source similar in spectral distribution to the one described here (see Methods), since the action spectrum of the light response is not known, and only for a photoperiod near 11½ light: 12½ dark, since effects of photoperiod have not been critically analyzed. The equation also assumes that the slope of the Y vs. log I curve, measured at 12° C (Figure 3.2), is independent of temperature, and the slope of the Y vs. temperature curve, measured at an intensity of 1.773×10^{13} photons$\cdot cm^{-2} \cdot sec^{-1}$ (not shown), is independent of light

intensity. Therefore the equation is expected to be less accurate the farther away the temperature and light intensity are from these values. Nonetheless, the level of %VP527 may be set within a wide range of values, even with these limitations.

In these experiments lower temperature and higher light intensity favored porphyropsin. These results are consistent with the visual pigment data collected by Allen et al. (1973) in the cutthroat trout, Salmo clarki, on the seasonal variations and differences between open and shaded stream areas, with the assumption that the seasonal variations are following the temperature cycle.

As further evidence that steady-state levels of %VP527 are different at different temperatures, consider the following data of rainbow trout kept in constant darkness. At 5° C the %VP527 fell from 55% to 40% after 5 weeks and fell no further during the next 10 weeks (Allen et al., 1973); at 4.5° C it fell from 50% to 43% after 4 weeks, and at 14° C it fell from 50% to 25% after 4 weeks (McFarland and Allen, 1973, and personal communication). The steady-state level is higher at the lower temperature.

A combination of temperature and light intensity that would have caused an increase of %VP527 from the initial group was not chosen in this study. However, Allen et al. (1973) have reported such cases with rainbow and brook trout in white light at 5° C.

The results of this report and the evidence cited above support the hypothesis of Allen et al. (1973) that seasonal temperature fluctuations are responsible for the seasonal visual pigment changes in salmonids. Possible mechanisms of temperature in setting steady-state

levels, such as activation of a vitamin A dehydrogenase at low temperature, are discussed by Allen et al. (1973) and Allen and McFarland (1973).

CHAPTER IV

DOSE-RESPONSE RELATIONSHIP OF OVINE PROLACTIN AND PORPHYROPSIN IN RAINBOW TROUT

The scotopic visual pigments of several species of Pacific salmon (Oncorhynchus) change from predominantly rhodopsin to predominantly porphyropsin during the spawning migration (Beatty, 1966). This transition also occurs in Atlantic salmon, Salmo salar, and apparently in sea-run rainbow trout, Salmo gairdneri (Beatty, personal communication). Since prolactin has been implicated in freshwater survival of a number of euryhaline fishes (see Lam, 1972), this hormone seemed a reasonable candidate for causing the visual pigment change in salmonids; and Cristy (1974) investigated the effects of ovine prolactin on the visual pigments of juvenile rainbow trout. In that study, the fish initially had a high percentage of porphyropsin. After hormone treatment, the prolactin-injected groups had a significantly higher percentage of porphyropsin than their control groups, but the control groups had decreased dramatically from the initial batch. The prolactin groups either increased slightly or decreased slightly from the initial batch, depending on dose. This state of affairs confounded the interpretation.

Recently I investigated factors which could have caused the large decrease in the control groups and found that both temperature and light intensity play a role in setting the steady-state percentage of porphyropsin in juvenile steelhead trout (Cristy, 1976). From that study the percentage of porphyropsin can now be manipulated to almost

any desired level before hormonal treatment; an empirical equation predicts the percentage porphyropsin to be reached after 4 weeks of a given temperature-light intensity regime (Cristy, 1976). Armed with this control over the rhodopsin/porphyropsin system, I have re-investigated the effects of ovine prolactin in juvenile steelhead trout.

In order to quantify a hormone dose-response relationship, some assurance is necessary that the animal's endogenous hormone production is zero or at least very low. For prolactin studies, the method of choice is hypophysectomy, but experimentally simpler methods have proven satisfactory in some cases, such as the prolactin bioassay of Clarke (1973), in which intact Tilapia are kept in seawater. In juvenile salmonids, hypophysectomy is impossible or at best very difficult, because of the location of the pituitary with respect to the gill arches (Neuman, 1974; Donaldson and McBride (1967) were successful with adult rainbow trout, but other workers have not been (Donaldson, personal communication)). However, the eta (prolactin) cells of the adenohypophysis may be inactive in juvenile Pacific salmon (Nagahama and Yamamoto, 1970; but see Discussion). The genera Salmo and Oncorhynchus are closely related, the latter believed to have evolved from the former and, in fact, from a fish very much like the present-day Salmo gairdneri (Neave, 1958). Therefore, if the eta cells are also inactive in juvenile steelhead, a dose-response relationship with exogenous prolactin will be meaningful.

Three bioassays for teleost prolactin, two using the familiar sodium-retaining property of prolactin (Ensor and Ball, 1968; Clarke,

1973) and one based on xanthophore-dispersing activity (Sage and Bern, 1972), have been developed. The xanthophore-dispersing assay is probably invalid, however (Farmer et al., 1975), and the assay developed by Ensor and Ball (1968) requires a stock of hypophysectomized Poecilia latipinna, limiting its value for routine use. The more convenient assay of Clarke (1973), utilizing intact seawater-adapted Tilapia mossambica, has recently been used to compare the potency of highly purified Tilapia prolactin with ovine prolactin (Farmer et al., 1975). Their finding that Tilapia prolactin was only 40-50 times more potent than ovine prolactin in this assay was surprising in light of the 10^4-10^5 fold potency of a crude teleost prolactin extract estimated from the (now-invalid) xanthophore-dispersing assay, a value that seemed believable at the time.

The bioassay to be suggested here is not intended to supplant the Clarke (1973) assay for routine use. About 50% more material is necessary for this assay, and the techniques for data gathering are more specialized and costly. However, it may be useful as an independent evaluation of the relative potencies of teleost and mammalian prolactins in fishes. Also, it could serve to test whether salmon pituitary material, which had no activity in Clarke's assay, is simply impotent in Tilapia but is potent in salmonids.

In order for a hormonal response to be useful for bioassay, the linear portion of the typically S-shaped response vs. log dose curve must be well-defined by testing a wide range of doses in preliminary experiments (Goldstein et al., 1974, pp. 89f; Finney, 1964, sections 3.5 and 3.9); this concept is much abused. In the experiments reported here,

I have attempted such a preliminary analysis. The range of doses to be tested was imperfectly estimated, as will be seen in Figure 4.2, but the log linear region can be estimated using symmetry arguments.

Materials and Methods

1. Fish

Juvenile rainbow trout (Salmo gairdneri, steelhead race), average weight 36 grams (range 14-71 grams), were obtained from the Leaburg Trout Hatchery, Leaburg, Oregon, on April 4, 1975. Water temperature at the hatchery was 5° C.

2. Experimental Conditions

The fish were brought into an experimental coldroom, and ten fish were immediately dark-adapted for sacrifice (initial$_1$ group, Table 4.1). The light intensity and temperature were set to a combination known to reduce the percentage of porphyropsin to a low value (Cristy, 1976).

The experimental facilities and spectral distribution of the dim "blue" light source are described by Cristy (1976). The average light intensity just above the water surface was 1.77×10^{13} photons$\cdot$cm$^{-2}\cdot$sec^{-1} [410-700 nm]. The light-dark cycle was 13:11, close to the outdoor photoperiod when the fish were acquired. The temperature was gradually increased to 12° C over several days and held within $\pm$ 0.5° C thereafter. The fish were fed lightly with trout chow (Sterling Silver Cup) to minimize fighting. Calcium chloride was added to the water at a concentration of 0.5 mM to reduce mortality.

After the fish had been held under these conditions for 33 days, another 11 fish were sacrificed for analysis of their visual pigments ($initial_2$ group, Table 4.1). Hormonal treatment was begun after another 1.5 days.

3. Hormonal Treatment

Ovine prolactin, NIH-P-S11, 26.4 IU/mg, a generous gift of the National Institute of Arthritis and Metabolic Disease, was used in this study. The hormone was dissolved in 0.6% NaCl, 0.5% $Na_2HPO_4 \cdot 7H_2O$, pH adjusted to 8.5 (first 5 daily injections) or 0.9% NaCl, pH adjusted to 9.0 (thereafter). The vehicle was changed and the handling procedure was improved after the fifth injection because the epidermis of the fish was losing its protective mucus. These changes had no obvious effects on the experimental results (see Figure 4.1). Mortality was a problem during the last week of the experiment, as can be seen by the reduced sample sizes at 14.5 and 18.5 days (Table 4.1).

Fish were injected intraperitoneally each day with a dose of 1, 3, 10, or 30 μg/g body weight or with the saline vehicle (volume 0.1 ml/10g body weight). Body weight for each fish was estimated to the nearest 10 grams by comparing its length with a chart of body weight-length data constructed from an earlier experiment. Lengths were measured the first 5 days but estimated visually thereafter to reduce handling (see above). Estimated weights in each group were recorded each day, and fish were weighed at sacrifice, so that actual average doses could be calculated (Table 4.1).

Because the number of animals in the experiment was large, the daily injections spanned a considerable time (Table 4.1). To test whether the time of day the injection was given affected the results, the 10 μg/g group was split in half, half being injected at 2½ and half at 7½ hours after onset of the light period. An additional 10 μg/g group, not used in the dose-response comparisons, was injected at 12 hours after onset of the light period.

Fish were given 5, 10, 15, or 19 injections and sacrificed approximately 12 hours after the last injection (Table 4.1).

4. Analysis of Visual Pigments

Fish were dark-adapted at least 2 hours before the retinae were removed. The 2 retinae of each fish were pooled to make one sample. Extracts were prepared and analyzed as described by Cristy (1976). $VP527_2$ or VP527 refers to trout porphyropsin.

5. Statistical Tests

Statistical tests were performed with model I analysis of variance (Sokal and Rohlf, 1969). The arcsine transformation was used to correct for non-homogeneity of variance (Finney, 1964, pp. 75-80).

Results

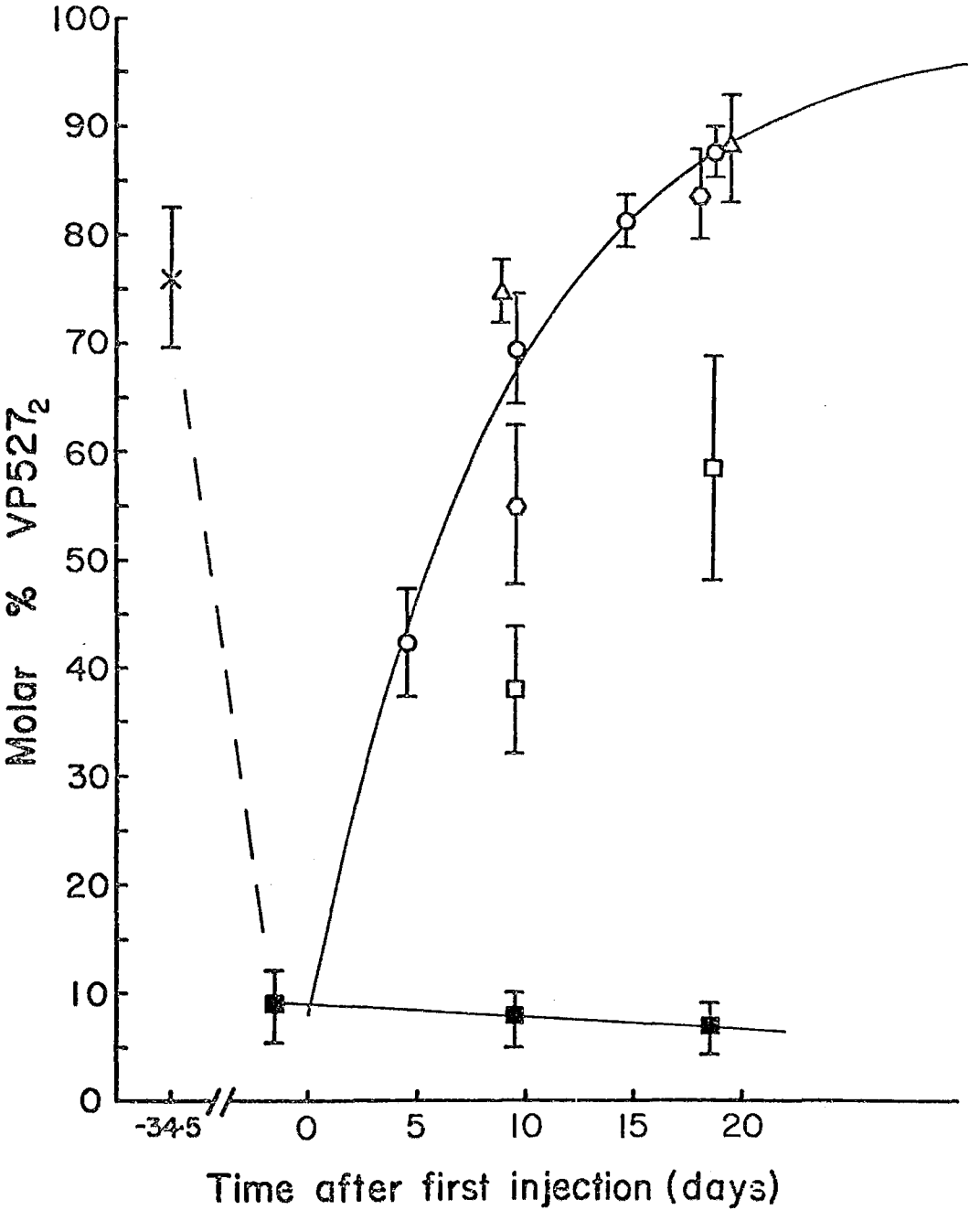
1. Timing of Response

When acquired from the hatchery, the trout had a high percentage of porphyropsin, but the %VP527 fell to a low level after 33 days under the experimental conditions, as expected (Figure 4.1). The %VP527 fell slightly in the control groups during the hormone treatment (9.1 to 7.8

Table 4.1. Data from entire experiment. The adjacent half-groups marked with an asterisk are combined in Figures 1 and 2.

nominal dose PRL (μg/g/day)	actual dose PRL	time after first PRL injection (days)	Number of injections	time injections given (hours after lights on)	Number of animals	Mean molar $\%VP527_2$	95% confidence limits of mean
$INITIAL_1$	—	-34.5	—	—	10	76.0	69.6 - 82.6
$INITIAL_2$	—	- 1.5	—	—	11	9.1	5.4 - 12.2
10*	10.5	4.5	5	2½	6	43.2	33.9 - 52.7
10*	11.2			7½	6	41.6	33.9 - 49.3
0	0	9.5	10	5½	12	7.8	5.0 - 10.1
1	1.13			3½	12	38.2	32.2 - 44.0
3	3.39			6½	11	55.1	47.8 - 62.6
10*	10.5			2½	6	66.8	58.9 - 74.5
10*	11.2			7½	6	72.3	63.6 - 80.8
10	12.2			12	10	67.9	63.7 - 72.3
30	34.2			4½	12	75.0	72.1 - 78.0
10*	10.5	14.5	15	2½	1	77.9	—
10*	11.2			7½	6	82.2	79.8 - 84.6
0	0	18.5	19	see	7	6.8	4.3 - 9.2
1	1.13			above	10	58.8	48.4 - 69.1
3	3.39				9	84.0	80.0 - 88.3
10*	10.5				5	89.1	86.9 - 91.2
10*	11.2				5	86.9	81.0 - 92.3
10	12.2				12	87.0	85.4 - 88.7
30	34.2				5	88.6	83.4 - 93.3

Figure 4.1.--Time course of response to prolactin. Fish acquired from the hatchery had a high percentage of porphyropsin (X). The experimental temperature and light intensity caused a reduction to low percentages, which was maintained in the saline-injected fish (filled squares). When the fish were injected daily with prolactin at doses of 1.13, 3.39, 10.9, or 34.2 μg/g (open squares, hexagons, circles, and triangles, respectively), the %VP527 increased significantly in each group. The half-time of response was estimated at 5.3-6.3 days; the solid curved line represents the equation $Y = (100\% - 8\%)(1 - e^{-0.11t}) + 8\%$ (see text). The vertical lines represent 95% confidence limits of the means.


100
90
80
70
60
50
40
30
20
10
0
Molar % VP527$_2$
-345
0
5
10
15
20
Time after first injection (days)

to 6.8%), but this trend was not statistically significant.

When trout were injected daily with 10.9 μg/g prolactin (average), their %VP527 rose exponentially, reaching 88.0% after 18.5 days (open circles, Figure 4.1). The half-time of response $t_{\frac{1}{2}}$, was calculated from the slope of the $\ln\left(1 - \frac{Y - Y_0}{Y_\infty - Y_0}\right)$ vs. time plot ($t_{\frac{1}{2}} = .693/\text{slope}$), where Y is the %VP527 at time t, Y_0 is the initial %VP527 (the average control value of 8% was used), and Y_∞ is the ultimate maximum. Judging from earlier work (Cristy, 1974), the %VP527 may never reach 100% in trout; in groups of trout achieving 92-93% porphyropsin after 17 days of treatment with very high doses of thyroxine, the variances were extremely small, even after the arcsine transformation was applied, suggesting that the maximum attainable is less than 100% (Finney, 1964, p. 79). When the data of Figure 4.1 (open circles) were plotted as above for several choices of Y_∞, the linearity was adequate for Y_∞'s between 95 and 100% but was poor for a Y_∞ of 92.5%. Because of this uncertainty of the Y_∞, the $t_{\frac{1}{2}}$ was estimated as 5.3-6.3 days, corresponding to Y_∞'s of 95-100%. The curved solid line in Figure 4.1 was drawn according to the equation $Y = (100\% - 8\%)(1 - e^{-0.11t}) + 8\%$, corresponding to a Y_∞ of 100% and a $t_{\frac{1}{2}}$ of 6.3 days.

2. Time of Day

Meier (1972) has reported that, in a wide range of animals, responses to prolactin injections are a function of the time of day the prolactin is administered. Since the injections given in this experiment necessarily spanned a considerable time, it was desirable to test whether

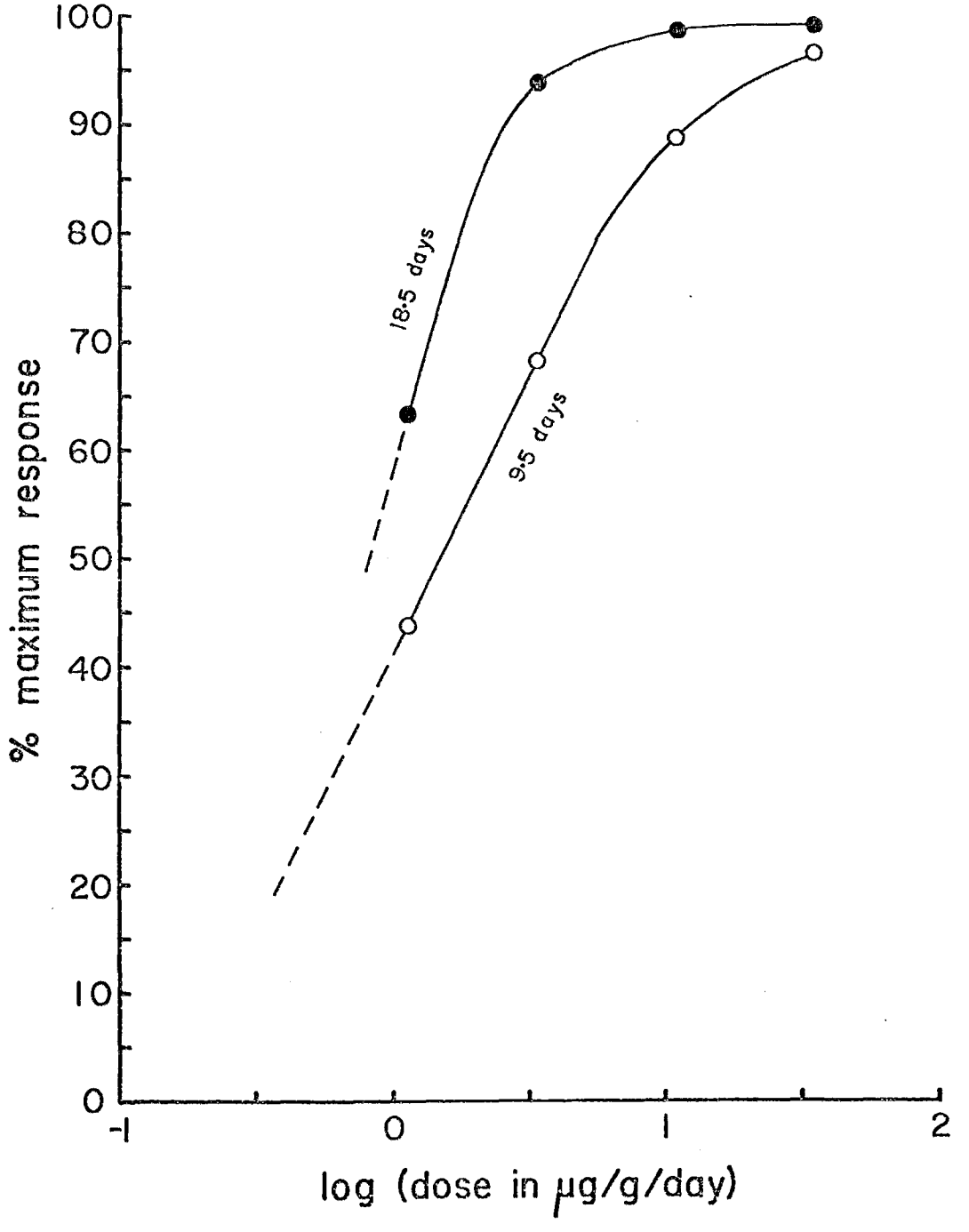
the time the prolactin was administered affected the response in this system. Therefore, the nominal dose of 10 μg/g/day was administered at 2½, 7½, or 12 hours after onset of the light period (Table 4.1). There was no significant difference among these three groups after 9.5 or 18.5 days, even when the group means were adjusted to take into account the small differences in actual average doses.

3. Dose-Response Relationship

The data of Table 4.1 are replotted in Figure 4.2 as percentage of maximal response ($100 \times (Y - Y_0) / (Y_{max} - Y_0)$) vs. log dose, where Y is the response for a given dose, Y_0 is the response of the control, and Y_{max} is the maximum response possible with high doses in the given time (9.5 or 18.5 days). Y_{max} was estimated from a double-reciprocal plot.

It is obvious from inspection that the dose range, estimated from the sodium-retaining response of teleosts to ovine prolactin (Ball and Ensor, 1967; Ensor and Ball, 1968; Utida et al., 1971; Clarke, 1973) was chosen too narrowly. In planning a bioassay, definition of the linear region of the S-shaped response vs. log dose curve is essential to insure statistical validity of the assay (Finney, 1964). The middle of this region occurs at the ED_{50}, the dose at which the response is 50% of maximum (Goldstein et al., 1974, p. 90). From Figure 4.2, the ED_{50} estimated after 9.5 days of treatment is 1.5 μg/g/day; the ED_{50} after 18.5 days, estimated from extrapolation, is 0.8 μg/g/day. Assuming symmetry around the 50% point, the linear region for the 9.5 day curve can be roughly estimated as lying between the doses of 0.4 and

Figure 4.2.--Dose-response relationship, normalized to percentage of maximum response possible in the given time (see text).


100
90
80
70
60
50
40
30
20
10
0
% maximum response
18·5 days
9·5 days
-1
0
1
2
log (dose in µg/g/day)

6 μg/g/day. No estimate can be made for the 18.5 day curve.

Discussion

1. Prolactin Response

In the experiment reported here, the control group was properly maintained at a low percentage of porphyropsin before hormonal treatment began. The increase in %VP527 in response to prolactin injections was large and clear-cut in a light and temperature environment known to favor rhodopsin in juvenile trout (Figure 4.1).

In these juvenile trout, the percentage of porphyropsin can be manipulated by changing light intensity or temperature (Cristy, 1976, and Figure 4.1). In adult Pacific salmon, on the other hand, Beatty (1966) argues against seasonal light (photoperiod or intensity) causing the visual pigment transition directly, because the transition occurs in both spring-run and fall-run salmon. In amphibians (Rana species) in which thyroxine causes an irreversible change from porphyropsin to rhodopsin at metamorphosis (Wilt, 1959a), the visual pigments of the larvae will interconvert reversibly in response to light and darkness (Bridges, 1974). Whether the prolactin effect is irreversible was not tested; in adult Pacific salmon, which die after spawning, the question is moot. Whether the prolactin acts directly on the eye tissues, as does thyroxine in Rana tadpoles (Wilt, 1959b), has not been determined.

The suggestion that the visual pigments in adult steelhead trout and Pacific salmon change in response to increased blood levels of prolactin during the spawning migration is attractive. This idea fits with the report that the eta (prolactin) cells of chum salmon

(Oncorhynchus keta) are not activated until the fish reaches sexual maturity (Nagahama and Yamamoto, 1970). In juvenile trout and salmon, prolactin would not ordinarily be a factor, but the eye tissue already has sensitivity to the hormone. Analogously, gonadotropin is effective in developing sexual maturity precociously in juvenile salmon (Donaldson et al., 1972). Under this scheme, the visual pigments of juvenile steelhead trout respond to light and temperature treatment because these environmental stimuli ordinarily regulate the %VP527 in juveniles (see Cristy, 1976), and they respond to exogenous prolactin because the sensitivity to prolactin, which the eye tissues are ordinarily not exposed to until the spawning migration, is already developed. It is not suggested that this scheme is proved.

Indeed, the role (or lack of a role) of prolactin in juvenile salmonids in fresh water is unclear. Based solely on histological evidence, it appears that the eta cells are active in sockeye and kokanee (O. nerka) smolts and in coho (O. kisutch) fry and smolts but are inactive in chum (O. keta) fry (McKeown and Leatherland, 1973; Leatherland and McKeown, 1974; Leatherland and Lin, 1975; Nagahama and Yamamoto, 1970). (This difference among species seems reasonable, since chum fry begin their seaward migration soon after hatching.) Furthermore, in kokanee smolts adapted to distilled water, tap water, one-third sea water, or sea water for 30 days, the eta cells appear more active in the former two media than in the latter two (Leatherland and McKeown, 1974). However, in the same experiment, serum prolactin levels were significantly higher in one-third sea water and sea water than in distilled water or tap water. The authors interpret these data to mean

that prolactin usage is more rapid in the hypoosmotic media, causing the serum prolactin levels to be lower. It is difficult to understand the role of prolactin in this hypothesis: what is the hormone's function if the receptors have independent information on when to "use" it? The authors cite the data of McKeown and van Overbeeke (1972) as fitting their usage hypothesis--the serum prolactin levels and pituitary prolactin content of adult sockeye salmon during their spawning migration are lower when the fish are near the mouth of the river than when they are farther out to sea or farther upriver. An alternate interpretation, however, is that the pituitary prolactin was "dumped" into the circulation prior to collection near the mouth, the plasma prolactin was eliminated by ordinary metabolic means, and prolactin synthesis was not fast enough to have restored the pituitary content and plasma levels. This interpretation would not apply to the kokanee smolt experiment, however, in which the fish were adapted for 30 days to the different media.

The hypotheses that prolactin causes the transition of visual pigments in adult steelhead trout and in adult Pacific salmon and that prolactin plays a significant role in freshwater osmoregulation in juvenile salmonids may or may not be mutually exclusive. In juvenile salmonids in fresh water, prolactin may be just one of several factors influencing the rhodopsin/porphyropsin ratio, and in adult salmonids the sudden surge of prolactin into the circulation during the spawning migration may "overwhelm" the system, or there may be a synergistic action of sex hormones. This speculation applies to coho, chinook (O. tshawytscha), chum, and pink (O. gorbuscha) salmon and for steelhead

trout; all of these salmon species and probably trout undergo a transition of visual pigments during the spawning migration (Beatty, 1966, and personal communication). In chum and pink, which have a short juvenile freshwater phase and in which prolactin is less likely to play an early role (Nagahama and Yamamoto, 1970), only the latter half of the argument may apply. It does not apply to O. nerka (sockeye and kokanee), which has a high proportion of rhodopsin at all times during its life cycle (Beatty, 1966), possibly of visual advantage for its life in clear blue lakes. During evolution the eye tissues have apparently lost their ability to maintain high proportions of porphyropsin in this species.

2. Comparison with Sodium-Retaining Response

To compare the potency of ovine prolactin in this response with its potency in sodium retention in other teleosts, I have computed ED_{50} values from dose-response relationships already published (Table 4.2). Although the responses are different in kind, and the responses are from different species, in each case the ED_{50} is the dose at which 50% of the maximum response, of whatever kind, is reached. Therefore the ED_{50} value should be valuable for rough cross-study comparisons.

A further complication in these cross-study comparisons is that in no case is there any assurance that the dose-response relationship has reached steady-state conditions; in the experiment reported here it is certainly not true. In addition, in some studies a single injection was given, and in others, multiple injections. How serious are these differences? If we assume the following:

Table 4.2. Comparison of ED_{50} values of responses to ovine prolactin in teleosts. The ED_{50} values were estimated from a graph of % maximum response vs. log dose; the maximum response had been estimated graphically from a double-reciprocal plot.

Response measured	Source	Adjusted* ED_{50}	Adjusted* ED_{50} (total dose)
increase in % porphyropsin in intact juvenile Salmo gairdneri in fresh water	this report; after 9.5 days: after 18.5 days:	1.4 µg/g/day 0.8 (10 or 19 daily doses)	14.1 µg/g 14.3
plasma $[Na^+]$ -retention in hypophysectomized Poecilia latipinna in fresh water	Ball and Ensor (1967); Table 5 and Figure 2	3.5 µg/g (single dose)	3.5
same, but using different procedure	Ensor and Ball (1968); composite data from Tables 2 and 3 and Figure 1	6.4 - 10.9 µg/g** (single dose)	6.4 - 10.9**
plasma $[Na^+]$ -increase in intact Oryzias latipes in sea water	Utida et al. (1971); Figure 5 and text	5.3 µg/g (single dose)	5.3
plasma $[Na^+]$ -increase in intact Tilapia mossambica in sea water	Clarke (1973); Figure 3: Table 1:	5.0 µg/g/day 5.8 (3 daily "priming" doses of 2 µg/g, followed by 2 daily test doses)	10.0 11.6

*Since the potencies of the ovine prolactin, in IU, varied with the studies, ED_{50} was rescaled to a "standard prolactin" with 28 IU/mg.

**There was no clear maximum response predicted by the double-reciprocal plot, because the response at the highest dose was out of line with the other data points when plotted on a double-reciprocal scale or a response-(linear) dose scale. Therefore a range of possible maxima was estimated.

i) the response vs. (linear) dose curve is hyperbolic (at steady state);

ii) the response vs. time curve has simple kinetics (zero- or first-order), and the rate constant is independent of dose; and

iii) if multiple injections are given, the biological half-time of the hormone is short compared with the inter-injection interval (i.e., no accumulation);

then the following will be true:

Even if a steady-state response has not been reached, the response vs. dose curve will still be hyperbolic (a constant fraction times the steady-state curve). Therefore, if the maximum response at a given time is measured directly or estimated from a double-reciprocal plot and the data are rescaled to per cent of this maximum, the curve will be identical to the curve at steady-state when similarly rescaled. This is true whether the abscissa is dose or log dose, of course. Therefore the ED_{50} value is independent of whether steady-state has been reached. The common observation (e.g., Clarke, 1973; Bates et al., 1963) that the slope of the response vs. log dose curve is increased by increasing the number of injections, when response is measured in raw units, is a trivial consequence of this idea. Consequently, if the response vs. log dose curve at any given time is known, and the kinetics are known, the slope of the response vs. log dose curve at any other time can be predicted. This is a rational approach to developing a bioassay, since the slope directly affects the index of precision of the assay (i.e., given a standard error, choose a slope to yield desired

index of precision). (If assumption iii is violated, the slope of the per cent maximum response vs. log dose curve also increases with time, and the whole curve is shifted to the left; and therefore the estimated ED_{50} will decrease with time.)

How valid are these assumptions for the data in Table 4.2? The first two assumptions are probably true, based on theory developed by Goldstein et al. (1974, chapters 1 and 4). The third assumption requires knowledge of the biological half-time of ovine prolactin in trout at 12° C and in Tilapia at 26° C; unfortunately we do not have this knowledge. Figure 4.2 suggests that the hormone may be accumulating in the trout, since the two curves are clearly not superimposable, contrary to prediction. If accumulation is significant, the real ED_{50} will lie between the ED_{50} based on daily doses and the ED_{50} based on total dose (last two columns of Table 4.2). However, even with this added uncertainty, the order of magnitude of the ED_{50} values can be compared. They are remarkably similar, ovine prolactin having roughly the same potency in several teleosts measured by different workers using different techniques and utilizing different responses.

3. Potential for Bio-Assay of Teleost Prolactin

The visual pigment response of juvenile trout to 10 daily injections of prolactin may be useful as a bio-assay for fish prolactin (Figure 4.2). The linear region of the response vs. log dose curve is estimated to be between 0.4 and 6 μg/g/day. The index of precision for the assay is approximately 0.3, similar to that in the Tilapia assay (Clarke, 1973). Although 10 daily injections are given, the amount of

material used is only about 50% more than in the Tilapia assay, because less material per injection is used.

The major weakness of the proposed assay at the present is the lack of knowledge of the effect of other hormones on the assay. The Tilapia sodium-retaining assay has the advantage of a decade of testing of specificity (Clarke, 1973, and references cited therein). The trout assay would be affected by TSH (Jacquest and Beatty, 1972), so that a test substance must be TSH-free or an anti-thyroid agent must be used with the assay. The effect of other hormones on the assay must be tested also, of course.

The validity of a bioassay also depends on the test and reference substances having the same maximum response at high doses (i.e., the same efficacy or intrinsic activity); then the relative potencies (=affinities) may be estimated from the amount of separation of the parallel response vs. log dose curves (see Goldstein et al., 1974, p. 99). The Tilapia assay does not suffer from mimicking or interfering responses of other pituitary hormones, and the parallelism of the response vs. log dose curves of the test substances and reference substance (ovine prolactin) seems to be satisfactory (Clarke, 1973). However, from Figure 3 of Clarke (1973), it appears that the Tilapia assay may lack fundamental validity, since the response to Cichlasoma rostral pars distalis appears to be greater than the maximum possible response to the reference material, ovine prolactin (maximum estimated from extrapolation of the double-reciprocal plot). A strong conclusion from extrapolation is dangerous, but it serves to illustrate that the fundamental validity of this assay has not been firmly established (see

also Table I of Clarke (1973) for a less clear-cut example of this doubt). The question of the intrinsic activities of ovine prolactin and teleost prolactins in this assay needs to be examined critically.

The proposed assay, should validity be established, is intended as an independent test of the relative potencies of teleost and mammalian prolactins in fishes and as a test of whether salmonid pituitary material, inactive in the Tilapia assay (Clarke, 1973), lacks prolactin activity in salmonids.

CHAPTER V

VISUAL PIGMENT PROPORTIONS IN TWO MIGRATORY POPULATIONS OF THREESPINE STICKLEBACKS --A MIXED POPULATION AND A PURE TRACHURUS POPULATION

The scotopic visual pigments of several species of Pacific salmon (Oncorhynchus) change from primarily rhodopsin to primarily porphyropsin during the spawning migration (Beatty, 1966). A similar change occurs in Atlantic salmon, Salmo salar, and apparently in steelhead trout, Salmo gairdneri (Beatty, personal communication). It has been suggested that changeable proportions of rhodopsin and porphyropsin may be of visual advantage to freshwater fishes, since freshwater photic environments are extremely variable, seasonally and over geological time periods (Munz, 1965). This hypothesis has been extended to the anadromous salmonids (Beatty, 1966): offshore oceanic waters are blue-green in color, whereas fresh waters are typically turbid and hence highly colored (green to brown) during the spawning migration. But it has also been questioned; for opposing viewpoints in the same volume, see Lythgoe (1972a) and Bridges (1972).

Some populations of the three-spined stickleback, Gasterosteus aculeatus, are anadromous. Freshwater G. aculeatus from California has both a rhodopsin and a porphyropsin (Munz, 1957a), but visual pigments of the anadromous form have not been studied. The work reported here was an attempt to establish whether the anadromous stickleback undergoes

a succession of visual pigments during the spawning migration similar to that in salmonids.

In the course of collecting sticklebacks for this study, I discovered that the morphology is highly complex. A literature search revealed that the morphology, taxonomy, nomenclature, and even life history of populations of Gasterosteus aculeatus are complex, confusing, and often the subject of controversy. This complexity is often ignored by physiologists, but it may be important in interpreting some physiological work.

Three morphological types are often recognized, based on the lateral bony plates: 1) "trachurus," which is fully plated; 2) "leiurus," which has plates only along the anterior flanks; and 3) "semiarmatus," which has anterior plates, a gap with no plates, and keeled plates on the caudal peduncle. The use of some of these terms to indicate subspecific or specific status, and the appropriateness of these European terms for Pacific sticklebacks, is a matter of contention (Hagen, 1967; Miller and Hubbs, 1969; Hagen and McPhail, 1970). Their use here is to indicate morph type only, without taxonomic implication, after Hay and McPhail (1975). These terms will be used without italics to emphasize this use.

In Europe pure trachurus populations are found in the extreme north and in the Black Sea; these populations are anadromous (Münzing, 1963). Along part of the Baltic coast and along the North Sea coasts and extending into the English Channel are mixed populations consisting of trachurus, semiarmatus, and leiurus in varying proportions; these fish are anadromous, but reproduction may take place in rather brackish

water as well as fresh water (Koch and Heuts, 1943; Heuts, 1947a; Münzing, 1963; van Mullem and van der Vlugt, 1964). Confusion with respect to these mixed populations has been aided by a misstatement made by Heuts (1947b) concerning data published in his 1947a paper in a less generally available journal (e.g., see Hagen and McPhail, 1970, p. 149, and Benjamin, 1974, p. 69). He stated on page 90: "Individuals intermediate between these modes [of the trachurus and leiurus forms] do occur, but they are not frequent in any populations." Actually, if the data from all of the Belgian mixed populations studied by Heuts (1947a, pp. 61-62) are combined, the intermediates (i.e., the semiarmatus morphs) outnumber the trachurus individuals: The populations comprised 41% trachurus, 50% semiarmatus, and 9% leiurus (n=1975). (Note: lateral plate numbers were given by Heuts, but no delineation into trachurus, semiarmatus, and leiurus was given. To get the above percentages, I have assumed that trachurus have 29-36 plates, semiarmatus have 9-29 plates, and leiurus have 4-9 plates (see p. 38 of Heuts). Therefore these percentages are not exact, but they should be close enough to establish the point.) A careful reading of Heuts (1947a) revealed that the misstatement was a semantic problem: he considered the grouping of the intermediates under the name semiarmatus to be arbitrary, and the quoted statement refers to the paucity of individuals <u>at any given intermediate plate number</u>, say 15. In any case the statement has been misinterpreted by several authors (including me!).

Permanently freshwater populations often consist of leiurus only, but two exceptional lake populations, one consisting of a mixture of trachurus and semiarmatus morphs and the other consisting of semiarmatus

only, have been reported (Münzing, 1963). Furthermore, pure trachurus populations are found far inland in eastern Europe, and these are presumably permanent freshwater residents (see Figure 1 of Münzing, 1963).

Along the Pacific coast of North America the situation is also complex. Morphology among freshwater populations is highly variable and complex (Miller and Hubbs, 1969; Hagen and Gilbertson, 1972). Excluding the freshwater populations, there are two types of populations of G. aculeatus in British Columbian waters: 1) pure trachurus populations, which appear suddenly in great numbers in coastal streams in the spring and are definitely anadromous; and 2) mixed populations (McPhail, personal communication). The latter type of population is found commonly in coastal waters around wharves and in eel grass beds (Hart, 1973). Its life history has not been investigated critically, but these fish are able to breed in rather brackish water (McPhail, personal communication). Thus the degree of anadromy within this population is uncertain. The location of the former populations in winter is somewhat a mystery: they may be far out to sea, based on occasional collections of large numbers of sticklebacks there (LeBrasseur, 1964; McPhail, personal communication); or they may be in coastal waters, at depth off underwater cliffs, based on their sudden appearance there in large numbers in August (LeBrasseur, personal communication). Plate morphology of the animals in these collections was not of interest and not analyzed, and these areas have not been trawled in the winter.

In the physiological literature this complexity is often ignored, either because it is considered unimportant or it is misinterpreted.

For example, in a series of physiological and anatomical studies on seasonal changes in activity of the prolactin cells in the pituitary, the experimental animals were consistently described as the anadromous, fully plated "Gasterosteus aculeatus L., form trachurus," collected in the coastal waters around Vancouver, B.C. (Lam and Hoar, 1967; Lam, 1968, 1969a,b; Lam and Leatherland, 1969a,b, 1970; Leatherland, 1970a,b, c,d; Leatherland and Lam, 1969a,b, 1971; and fish were also collected in the Campbell River in two of the reports by Leatherland, 1970a,b). The reader's impression is that the fish were from a pure trachurus population. However, in his Ph.D. thesis Lam stated: "Furthermore, many fish showed a reduced number of lateral plates extending only up to the mid-flank. These fish may be hybrids between the marine form and the freshwater form (leiurus) [but see below, under Results], and were also not used in the studies" (Lam, 1969c, in "Materials and Methods"). Thus the fish used by Lam and Leatherland were trachurus morphs selected from the second type of population above, with the possible exception of the Campbell River fish studied by Leatherland (1970a,b). If the Campbell River fish were from a pure trachurus population, it may be unfair to compare them with trachurus morphs from a mixed population to gain insight on seasonal changes in pituitary anatomy, as Leatherland has done. For example, there may be a difference in the (average) salinity of the spawning grounds of the two kinds of populations (prolactin is often implicated in freshwater osmoregulation in euryhaline fishes--see reviews by Ball, 1969; Lam, 1972; Johnson, 1973). The physiology of the two types of populations may differ in detail, as the genetics of plate morphology apparently does: results of laboratory crosses between

trachurus and leiurus are different in detail, according to whether the parents are chosen from pure trachurus and pure leiurus populations from the Pacific coast (Hagen, 1967, Fig. 10) or from a mixed population and a pure leiurus population from Europe (Münzing, 1959, Fig. 10 D,F). On the other hand, it is probable that Lam and Leatherland are correct in their overall assessment of the seasonal prolactin cell changes, anyway, for most of the physiological work and anatomical work was done with trachurus morphs (apparently) selected from mixed populations. These mixed populations probably spawn in both brackish and fresh waters, as do the European mixed populations and the Coos Bay, Oregon, mixed population (this report).

Thus the situation is much more complex than commonly presented in the physiological literature. The physiology of trachurus from homogeneous populations may or may not prove to differ significantly from that of trachurus from mixed populations; but the failure to state clearly the source of the experimental animals (e.g., series by Lam and Leatherland; Wendelaar Bonga, 1973a,b; Wendelaar Bonga and Veenhuis, 1974a,b) further confuses an already difficult literature.

Two populations of sticklebacks were used in this study: a mixed population from the Coos Bay estuary, Oregon, and a pure trachurus population from the Rogue River, Oregon. In addition, a few sticklebacks were collected in British Columbian waters in winter, some from a mixed population and some from a population of uncertain morphology (see Results).

Materials and Methods

Fish

Coos Bay, Oregon

Sticklebacks were caught with seine or dipnet at the numbered locations shown in Figure 5.1. The water of Isthmus Slough is brackish in summer, 7 ‰ salinity measured at location 1 at low tide, but in winter and early spring the salinity is less than 1‰, also measured at low tide. At locations 2 and 6, the water is fresh all year. The collections comprised leiurus, semiarmatus, and trachurus morphs. Only adults were studied, except for the January 1975 collection. For leiurus and semiarmatus morphs, the number of anterior lateral bony plates (i.e., excluding the keeled caudal plates for semiarmatus) was counted on the left side, using a probe but no magnification. The morph type was also recorded. Standard length was routinely recorded for fish sacrificed for visual pigment analysis.

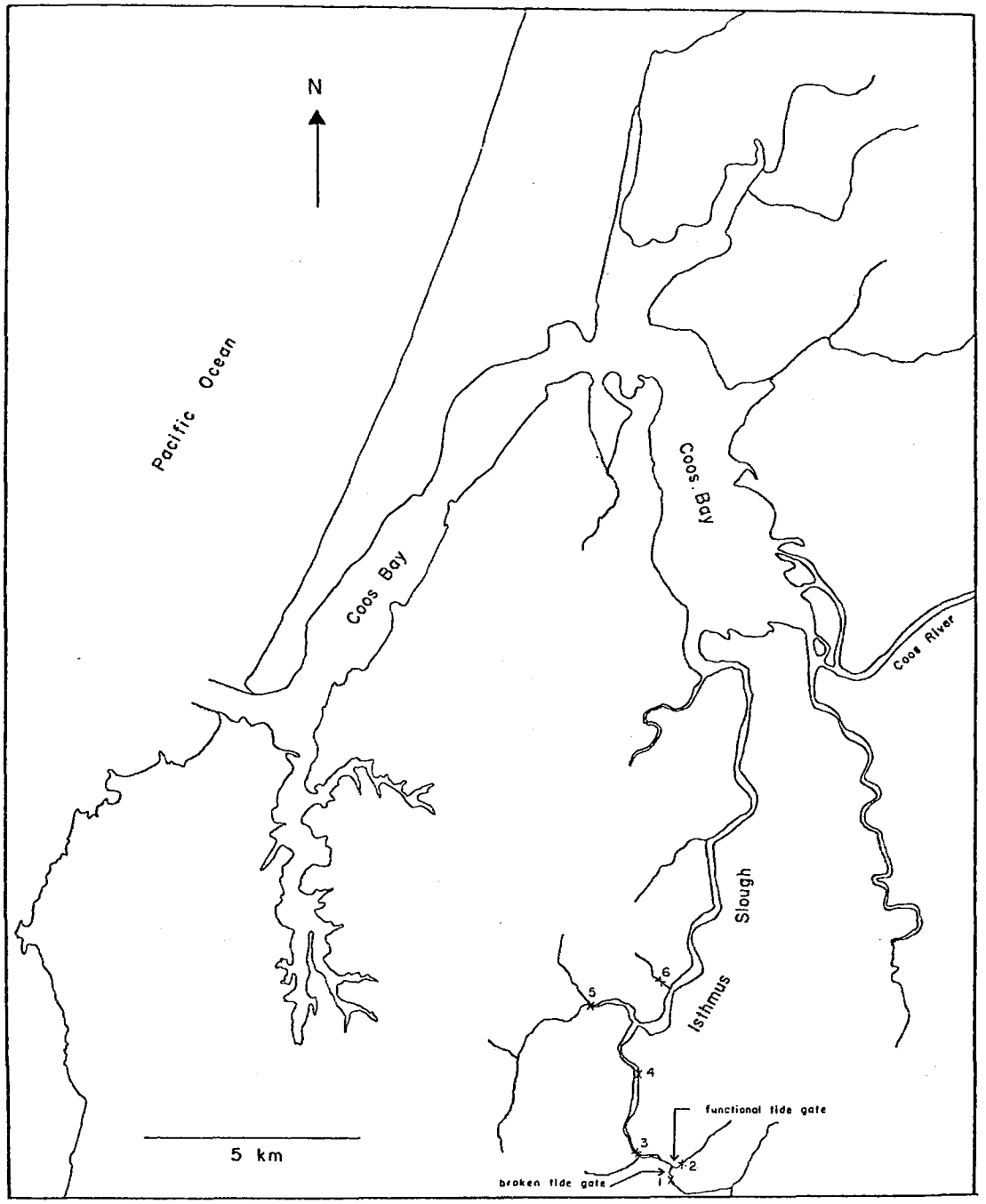
Rogue River, Oregon

Sticklebacks were caught during the spring and summer of 1974 and 1975 at various locations from river mile (R.M.) 0.5 to R.M. 27 (given in tables and figures). Fish were caught by trap or seine. All fish were trachurus morphs, and only adults were studied.

British Columbia

Sticklebacks were caught by dipnet around floats in Horseshoe Bay (near Vancouver) and by purse seine in deep water of Departure Bay (near

Figure 5.1.--Map of Coos Bay and Isthmus Slough, showing locations where sticklebacks of the mixed population were caught.


N
Pacific Ocean
Coos Bay
Coos Bay
Coos River
Slough
Isthmus
6
5
4
3
2
1
functional tide gate
broken tide gate
5 km

Nanaimo) in early January 1975. The morphology is discussed in Results. All were juveniles.

Visual Pigment Analysis

Fish were kept in light-proof cans from collection until sacrifice on the same night or the following night. The two retinae of individual fish were pooled to make one extract, unless noted otherwise. Retinal extracts were prepared using standard methods as described in Allen et al. (1973), except that 0.5 ml of 2% digitonin and 0.05 ml of saturated sodium borate were routinely used. In some of the early work, the volumes were 60% greater.

The method of partial bleaching with monochromatic lights was used to determine the λ_{max}'s of the visual pigments (see Dartnall et al., 1961; Munz and Beatty, 1965). The bleaching apparatus employed was the second one described by Munz and Beatty (p. 4). For bleaching, light from a ribbon-filament tungsten lamp was passed through interference filters (Baird-Atomic, Incorporated). Filters with transmission maxima at 680, 675, 660, 610, and 560 nm were used; the half band widths were about 4 per cent of λ_{max}, except for the 680 nm filter, whose half band width was 1.5 per cent of λ_{max}. Spectra were obtained with a Cary 14 recording spectrophotometer, equipped to record absorbance data on magnetic tape, and difference spectra were calculated and drawn by computer (Munz and Allen, 1968).

Many of the extracts not used for λ_{max} determinations were bleached in one stage with 610 or 560 nm light. The percentage of porphyropsin in each extract was determined by the method of template

curves developed by Dartnall et al. (1961; see also Munz and Beatty, 1965, and Munz and Allen, 1968). Template curves were calculated by a computer program of mine, which generates template curves for a mixture of any two visual pigments, given only the λ_{max}'s and the types of prosthetic groups. The theoretical basis of this program is that the absorbance spectra of all rhodopsins have the same shape when plotted against frequency rather than wavelength (Dartnall, 1953); the same is true for all porphyropsins studied (Munz and Schwanzara, 1967; Bridges, 1967). The standard curves for rhodopsin and for porphyropsin for this program were constructed from the published nomograms (Dartnall, 1953, Table 1, but with λ_{max} corrected by subtracting 1.3 nm--personal communication to Munz, 1968; Munz and Schwanzara, 1967) and from detailed absorbance spectra for several rhodopsins and several porphyropsins, provided by Prof. F. W. Munz, all replotted on a frequency scale.

The percentages of porphyropsin were calculated using template curves for mixtures of the pigments $P500_1$ and $P522_2$, based on an early and incorrect guess of the λ_{max}'s of stickleback rhodopsin and porphyropsin. The λ_{max}'s are actually 499 and 524 nm, respectively, to the nearest nanometer (see Results). For the purpose of constructing standard curves, the λ_{max}'s were rounded to the nearest half-nanometer, i.e., 499.5 and 524.5 nm. Correction factors were calculated by the following method: Absorbance spectra for mixtures of $P499.5_1$ and $P524.5_2$ in various proportions were calculated, using the computer program described above. Then the "percentage of $P522_2$" was calculated for each of these mixtures, so that an additive correction factor could

be determined. Correction factors were calculated for mixtures containing 0,10,20, . . . , 80,90, and 100% $P524.5_2$; and a smooth correction curve was drawn. The correction factors varied from +3.0 to -11.3%. The percentage of $P524.5_2$ for each extract, calculated in this fashion, was then converted to molar percentage of $P524.5_2$, using the value 1.42 for the ratio of the photosensitivity of rhodopsins to that of porphyropsins (Dartnall, 1968).

Statistical Analysis

For the visual pigment data, the arcsine transformation was used to correct for non-homogeneity of variances before statistical tests were applied (see Finney, 1964, pp. 75-80), but means and their 95% confidence limits are reported in percentage units. Statistical tests were made by analysis of variance (Sokal and Rohlf, 1969) or with two-way analysis of variance; the latter analysis was performed with a library program (TWOAOV) of the University of Oregon Computing Center, which performs the analysis for the unbalanced case using the procedure outlined by Bancroft (1968, sections 1.7-1.9).

Results

Morphology

The plate counts and standard length measurements were made incidentally to the visual pigment study. Although large numbers of sticklebacks were caught at many locations, usually counts and lengths were determined only for the fish sacrificed for visual pigment analysis,

because the possible importance of the diversity was not appreciated during the first part of the study, when the Coos Bay fish were studied, and there was not much morphological diversity to measure in the Rogue fish, studied later. Paradoxically, the visual pigment (rhodopsin/porphyropsin) proportions proved to be diverse in the Rogue sticklebacks and uniform in the Coos Bay sticklebacks (see below). Nevertheless, the data are numerous enough in some cases to draw conclusions and are suggestive in other cases.

Coos Bay

The number of anterior lateral plates for various collections are given in Table 5.1. All of the adult fish caught at location 1 (see Figure 5.1) in July 1973 were brought to the laboratory for experiments, and plate counts were made on all of these fish, including ones which died or were not used in the experiments. Thus these plate counts should be representative of the location, unless the collecting technique was not random (and this is an important consideration, for Moodie (1972) reported that breeding sites and plate numbers were correlated for a peculiar freshwater stickleback population in Canada). This collection comprised 38% leiurus, 28% semiarmatus, and 34% trachurus (n=252).

Forty-to-fifty adult fish caught in January 1974 at location 2, a small stream above a tide gate, were all leiurus. Plate counts for the twenty fish brought to the laboratory are given in Table 5.1. These fish had plate counts similar to the leiurus morphs collected at location 1 the preceding July, but these fish were a mottled brown

Table 5.1. Counts of lateral bony plates and morph types. Only the anterior plates were counted; i.e., the keeled plates on the caudal peduncle of semiarmatus morphs were not counted. The map locations refer to Figure 5.1.

Date	Map location	Morph	Number of anterior plates																		Total
			4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
24 Jul 73	6	leiurus + semiarmatus[A]	1	1						1	1	1										5
		trachurus																				8
27 Jul 73	1	leiurus + semiarmatus[A]		3	2	3			1	2	1	1		1								14
		trachurus																				6
27 Jul 73	1[B]	leiurus		5	20	29	18	6	6	6	3	1		1								95
		semiarmatus					2	3	5	5	3	8	13	11	10	4	5		1		1	71
		trachurus																				86 (3)[C]
5 Jan 74	2	leiurus		1	6	3	3	4	1	1		1										20
		semiarmatus																				0
		trachurus																				0
19 Mar 74	3	leiurus						2														2
		semiarmatus														1						1
		trachurus																				9 (1)[C]
18 May 74	6	leiurus			2	3																5
		semiarmatus					1			1			3	2		2	2					11
		trachurus																				26 (1)[C]
18 May 74	5	leiurus			2	1				1	1											5
		semiarmatus									1	1	1		1		1					5
		trachurus																				23 (1)[C]
19 Jan 75	3 - 4	leiurus																				0
		semiarmatus														1						1
		trachurus																				8
spring-summer, 1974 and 1975	Rogue River, river miles 0.5 - 27	leiurus																				0
		semiarmatus																				0
		trachurus																				866

[A] No distinction was made between leiurus and semiarmatus at the beginning of the study. Also, one or two of the anterior-most, reduced plates may have been missed at this time.

[B] These fish were kept for a laboratory experiment and measured after sacrifice.

[C] Occasionally a fish had small gaps of 1-2 plates; these were scored as trachurus; number of these fish is given in parentheses.

color, whereas all three morphs of the July collection were greenish and lacked mottling. Unfortunately location 2 was not sampled in spring or summer. Thus their color during the breeding season is unknown, and the effectiveness of the tide gate against migration of the mixed population is unknown. Locations 1 and 6 were scoured with seine and dipnet in winter, but only one or two adults were found at each location. These were leiurus morphs and mottled brown. Several juveniles were also found, but they were too small for complete plate development (see Heuts, 1947c; Münzing, 1959; van Mullem and van der Vlugt, 1964; Hagen and McPhail, 1970) and were not analyzed.

At other locations (3-6), the data suggest that the proportion of trachurus morphs is higher than at location 1 (see Table 5.1). The diking and undiking near locations 1 and 2 (see Figure 5.1) may have affected the populations in these areas selectively. However, since the sample sizes are small, the samples were collected at different times of the year, and there is no assurance of randomness of the collection methods, no firm conclusion can be drawn.

An important question is: are the semiarmatus morphs hybrids between migratory trachurus and resident leiurus, or (with the exception of the mottled brown leiurus plentiful at location 2 but scarce at 1 and 6 in winter) do the three morphs comprise a single population, similar to the anadromous mixed populations in Europe (Münzing, 1959, 1963; van Mullem and van der Vlugt, 1964)? An anadromous, pure trachurus population and a freshwater, pure leiurus population in the Little Campbell River, British Columbia, have been studied in depth by Hagen (1967). These populations are allopatric except for a narrow hybrid

zone; they differ in a host of traits, including size and color. Trachurus individuals are larger and have a bright silver color; leiurus are a mottled olive color. Hybrids, collected in the hybrid zone or reared from crosses between trachurus and leiurus, are intermediate in the five traits used by Hagen for his hybrid index (lateral plates, gill rakers, electrophoretic patterns of muscle proteins, body depth into standard length, and standard length of adults, in decreasing order of usefulness). Although standard length was the least useful of the five traits for determining hybrid origin of individual fish, because of considerable overlap, the populations of trachurus and leiurus were clearly different in their means, and the hybrids were intermediate (Hagen, Fig. 5). Of the 252 fish seined in July 1973 at location 1 (Table 5.1), standard lengths were recorded for the 107 used in an experiment; these are given in Table 5.2. A two-way analysis of variance was performed to test between sexes and among morphs. The males were significantly ($p < .001$) smaller than the females, but there was no significant difference among the morphs. Similar results were reported by Münzing (1959) and van Mullen and van der Vlugt (1964) for anadromous mixed populations in Europe. These data, plus the uniform color of the fish, suggest a single population.

Rogue River

The Rogue sticklebacks were an anadromous, pure trachurus population (Table 5.1). Of 866 fish examined individually, all were trachurus. They were bright silver laterally and gray to bluish-black dorsally, and they were larger than the Coos Bay sticklebacks (Table 5.2).

Table 5.2. Standard lengths of Coos Bay and Rogue River sticklebacks, tabulated according to sex and morph type.

Date	Location	Morph	♂♂ Standard length ± S.E. (n)	♀♀ Standard length ± S.E. (n)	Statistical tests
27 Jul 73[A]	1 of Figure 5.1 (Isthmus Slough, Coos Bay)	leiurus	4.53 ± 0.10 cm (13)	4.83 ± 0.09 cm (28)	between sexes: $p < .001$; among morphs: not significant
		semiarmatus	4.46 ± 0.08 (12)	4.89 ± 0.10 (19)	
		trachurus	4.53 ± 0.16 (8)	4.83 ± 0.07 (27)	
19 May 74	Rogue, R.M. 10	trachurus	5.96 ± 0.03 (106)	5.87 ± 0.03 (120)	between sexes: $p < .05$
18 Apr 75[B]	Rogue, R.M. 3.5	trachurus	5.87 ± 0.08 (6)	5.67 ± 0.08 (16)	between sexes: not significant; among groups: $p < .001$
18 Apr	11.5	"	5.70 (2)	5.67 ± 0.11 (6)	
3 May	15	"	5.83 ± 0.09 (11)	5.75 ± 0.07 (13)	
12 May	0.5	"	5.88 ± 0.10 (5)	5.98 ± 0.13 (5)	
10 Jun	15	"	5.93 ± 0.10 (11)	5.78 ± 0.11 (13)	
10 Jun	27	"	6.04 ± 0.06 (9)	5.89 ± 0.08 (15)	
31 Jul	10	"	5.96 ± 0.05 (13)	6.01 ± 0.08 (11)	
31 Jul	19-22	"	——— (0)	6.16 ± 0.09 (12)	
31 Jul	25	"	6.00 ± 0.06 (13)	6.05 ± 0.07 (11)	

[A] The numbers of animals are fewer than the corresponding group in Table 5.1, because lengths were measured only for those animals whose visual pigments were analyzed, whereas plate counts were made for all animals.

[B] These lengths are of the fish whose visual pigment proportions are shown in Figure 5.6; numbers of fish do not agree exactly, because the sexes of 3 fish were not determined and 1 pigment extract was lost.

The males were larger than the females in a single large collection of May 1974, but the difference was trivial and the significance level low (Table 5.2). The test for sex differences was repeated for a number of smaller collections made from April through July, 1975, using two-way analysis of variance. For these data there was no significant difference between the sexes. There was a highly significant difference among the collections, however, which I interpret as simply continuing growth through the season. These results differ from those of Narver (1969, quoting a MS), who found the females significantly larger in a pure trachurus population in Alaska.

British Columbia

Of the 30 juveniles from Horseshoe Bay which were large enough for complete acquisition of plates to have occurred (see Heuts, 1947c; Münzing, 1959; van Mullem and van der Vlugt, 1964; Hagen and McPhail, 1970) and which were closely examined, 25 were trachurus, 4 were semiarmatus, and one was leiurus.

An attempt was made to collect sticklebacks in deeper water off cliffs in the winter, where LeBrasseur found them appearing in August (see Introduction), to determine whether these fish are from a pure trachurus or a mixed population, and to analyze their visual pigments. Through the kindness of R. J. LeBrasseur, I visited Nanaimo for several days in January 1975 for this purpose. Unfortunately, the weather was too severe to venture out into Stuart Channel, where the August collections were made, in the small boat available. Therefore a similar habitat in Departure Bay was seined. This habitat was much less ideal

for seining, however, because it was a burial ground for automobile bodies. Hence fishing depth was restricted to 8 fathoms for fear of injuring the purse seine. Ten juveniles were caught, all of which were trachurus morphs. But since the collection site was so poor and the number caught was so small, the questions of the abundance of sticklebacks in winter and the type of population in these habitats are still unanswered.

Visual Pigments

Absorbance Maxima

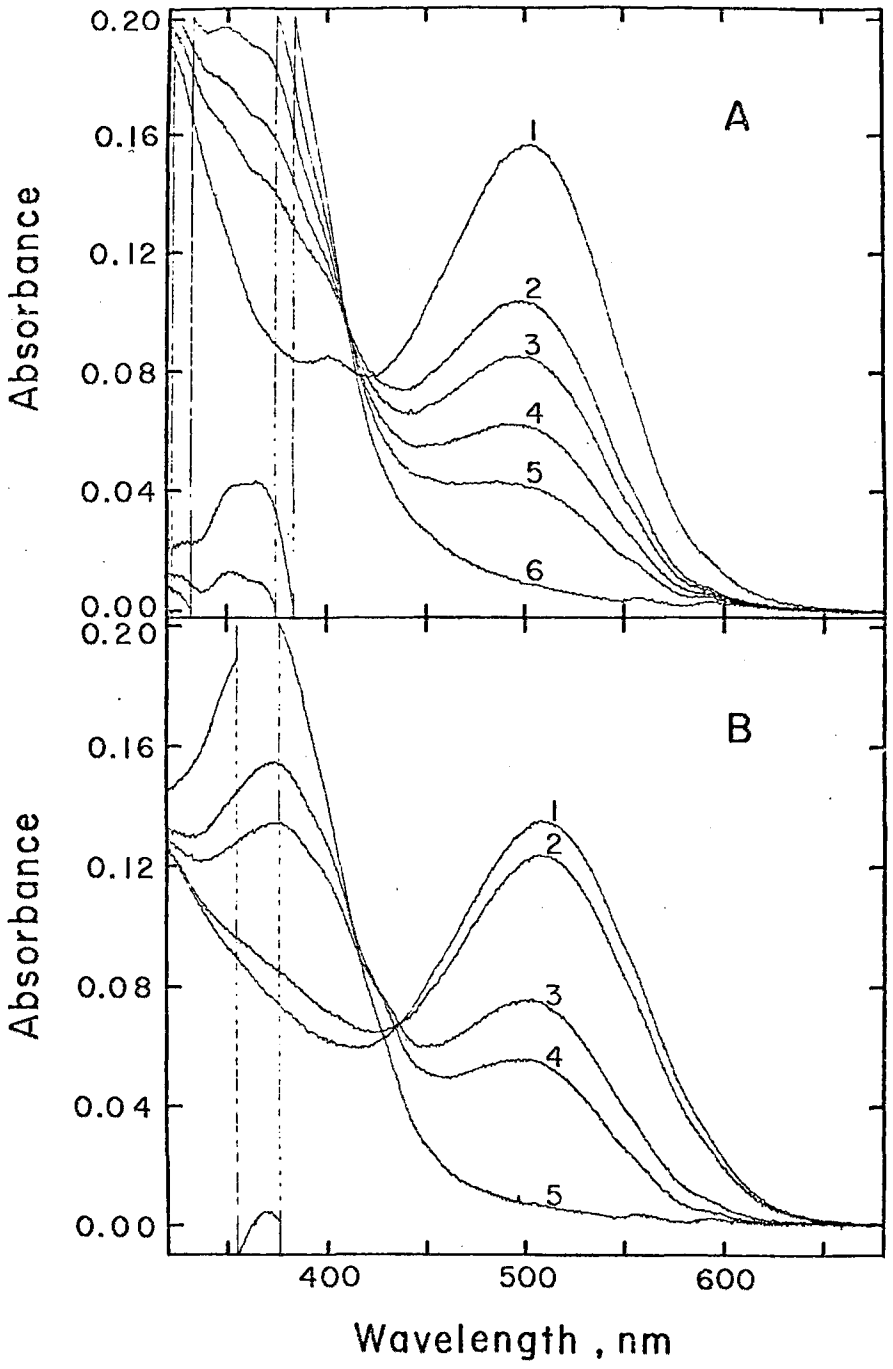
Only one previous study of the visual pigments of Gasterosteus aculeatus has been made, as a minor part of a Ph.D. thesis by Munz (1957a), but the data were never published. The study showed the presence of both a rhodopsin and a porphyropsin, but precise estimates of their λ_{max}'s could not be made. The λ_{max}'s are given in a list of vertebrate visual pigments compiled by Lythgoe (1972b) as 501 and 522 nm, but these values should not be taken seriously (Munz, personal communication) or to conflict with the values reported here.

Rhodopsin

The method for determining the λ_{max} of the rhodopsin was straightforward. All or virtually all of the porphyropsin (and some of the rhodopsin) was first bleached with 675 and/or 660 nm light (to be noted as "λ675" and "λ660," for brevity), and then the remaining rhodopsin was bleached in 1-3 stages with λ610 and/or λ560. The technique is illustrated in Figure 5.2A for experiment 4939. Curve 1 is the

Figure 5.2.--(A) Absorbance spectra for experiment 4939. Curve 1 is spectrum of unbleached extract; curve 2, after 3 min 45 sec bleach with λ660; curve 3, after another 3 min with λ600; curve 4, after 6 sec bleach with λ610; curve 5, after another 8 sec with λ610; curve 6, after 6 min bleach with λ560. The baseline on the spectrophotometer was routinely set to negative values below 400 nm to insure that the photoproduct curve would be on scale--see curve 1 at 400 nm. The absorbance scale is effectively twice that shown, for the recorder pen automatically shifts to the bottom of the chart when the top of the chart has been reached--see curves 5 and 6 below 390 nm.

(B) Absorbance spectra for experiment 4893. Curve 1 is spectrum of unbleached extract; curve 2, after 3 min 30 sec bleach with λ680; curve 3, after 4 min bleach with λ675; curve 4, after 6 min bleach with λ660; curve 5, after 6 min bleach with λ560.


A
B
Absorbance
Absorbance
0.20
0.16
0.12
0.08
0.04
0.00
1
2
3
4
5
6
400
500
600
Wavelength , nm

absorbance spectrum of the unbleached extract. Curves 2-6 were recorded after the following bleaches: 3 min 45 sec of λ660, 3 min of λ660, 6 sec of λ610, 8 sec of λ610, and 6 min of λ560, respectively. The difference spectra are shown in Figure 5.3A, and each is replotted as per cent of maximum in Figure 5.3B. The first difference spectrum (1-2) is of a mixture of porphyropsin and rhodopsin, but the final four spectra are of rhodopsin only, as is seen clearly by their near-superimposability in Figure 5.3B. That the pigment is a rhodopsin is established by the product of bleaching curve, typical of retinene$_1$ oxime.

For each of these final bleaches 7 estimates of the λ_{max} were calculated using Dartnall's nomogram (1953, with correction--see Materials and Methods) and the wavelengths at which the difference spectrum was 30,40, ... , 80, and 90% of maximum (along the long-wavelength arm), and the mean and standard error of these 7 estimates was computed. The means and standard errors are given in Table 5.3 (experiment 4939).

In all, 11 experiments were performed, from which λ_{max} estimates from 17 final bleaches were made (Table 5.3). (The bleaching protocols varied in detail: the initial bleaches with λ675 and/or λ660 were continued until all the porphyropsin was bleached.) The range of these mean estimates is from 498.0 to 501.3 nm. The range is this large because the extracts were dilute--individual fish were used, and sticklebacks are small. Thus, in a given experiment, any unidirectional drift in the baseline or error in setting the baseline before a spectrum is run will affect the mean λ_{max} estimate systematically, and the error will be greater the more dilute in the extract. But if many experiments

Figure 5.3.--(A) Difference spectra for experiment 4939.

(B) Difference spectra, rescaled to per cent of pigment absorbance maximum. The absolute values are plotted, so that the photoproduct curves appear here as positive-going. The symbols on the curves are used for identification only; the data points are at every 1 nm.

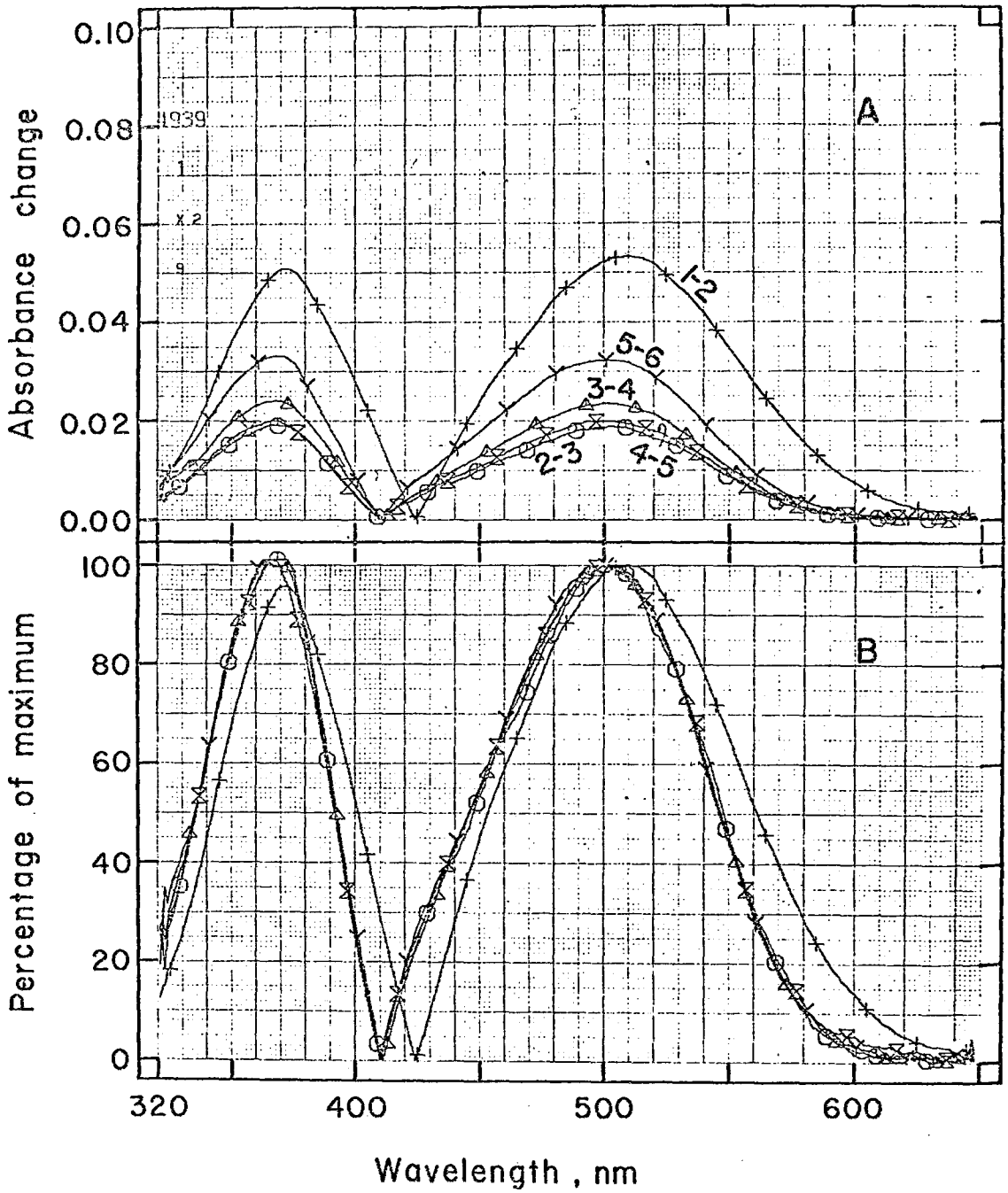

0.10
0.08
0.06
0.04
0.02
0.00
Absorbance change
1939
1
x 2
9
A
1-2
5-6
3-4
2-3
4-5
100
80
60
40
20
0
Percentage of maximum
B
320
400
500
600
Wavelength , nm

Table 5.3. Estimates of rhodopsin λ_{max}. See text.

Experiment number	Molar % porphyropsin in extract[A]	Absorbance at λ_{max}, total extract	$\lambda_{max} \pm$ S.E., final bleach	Absorbance at λ_{max}	$\lambda_{max} \pm$ S.E., bleach preceding final[B]	Absorbance at λ_{max}	$\lambda_{max} \pm$ S.E., antepenultimate bleach	Absorbance at λ_{max}	λ_{max}, bleach preceding final bleach(es)	Absorbance at λ_{max}
4915	26.9	0.076	500.2 ± 0.29	0.016	500.1 ± 0.14	0.018			500.7	0.018
4919	38.4	0.175	499.9 ± 0.43	0.034	499.2 ± 0.22	0.031			501.5	0.035
4921	24.6	0.117	499.0 ± 0.23	0.023	498.0 ± 0.14	0.030			500.6	0.028
4928	31.8	0.099	499.5 ± 0.18	0.043					511.6[C]	0.056
4930	63.2	0.127	499.6 ± 0.11	0.033					501.6	0.008
4931	52.5	0.076	498.5 ± 0.20	0.024					498.5	0.005
4932	33.2	0.142	499.4 ± 0.16	0.063					501.2	0.016
4933	57.8	0.151	498.7 ± 0.21	0.043					500.7	0.010
4937	52.0	0.088	501.3 ± 0.39	0.019	500.9 ± 0.23	0.011			517.7[C]	0.059
4939	22.3	0.147	499.5 ± 0.21	0.032	500.5 ± 0.21	0.020	499.9 ± 0.29	0.023	499.6	0.019
4941	61.5	0.113	498.0 ± 0.11	0.031					521.0[C]	0.084

[A] calculated after λ_{max}'s had been determined.

[B] i.e., the penultimate bleach.

[C] first difference spectrum; but bleach was sufficiently long to bleach out all the porphyropsin, based on experience from the other 8 experiments.

are run, these errors will cancel. Excluding the two means with standard errors greater than 0.30, the overall average of the means was 499.4 $\pm$ 0.2 nm (n=15).

Porphyropsin

The determination of λ_{max} for the porphyropsin was more difficult. The protocol is illustrated in Figure 5.2B for experiment 4893. Curve 1 is again the absorbance spectrum of the unbleached extract. Curves 2-5 were recorded after the following bleaches: 3 min 30 sec of λ680, 4 min of λ675, 6 min of λ660, and 6 min of λ560, respectively. The difference spectra are shown in Figure 5.4A, and each is replotted as per cent of maximum in Figure 5.4B. Much less pigment is bleached in the first bleach than in the second bleach (recall that the bandwidth of the 680 nm filter is smaller than that of the 675 filter; Materials and Methods).

For each of the initial difference spectra (1-2 and 2-3, Figure 5.4B), 7 estimates of λ_{max} were calculated as above for rhodopsin, except that the nomogram for porphyropsins (Munz and Schwanzara, 1967) was used. Another 8 experiments were performed, in which the initial bleaching protocols were identical--the means and standard errors of the λ_{max} estimates are given in Table 5.4 (columns 4 and 6).

Note that the mean λ_{max} estimates from the first bleach are consistently higher than the mean λ_{max} estimates from the second bleach. Also, the standard errors of the means from the first bleach are higher, because the amount of pigment is much less. In other experiments (not given), several successive bleaches with λ680 were made, but the same pattern emerged--the mean λ_{max} estimates decreased with each bleach.

Figure 5.4.--(A) Difference spectra for experiment 4893.

(B) Difference spectra, rescaled to per cent of pigment absorbance maximum.

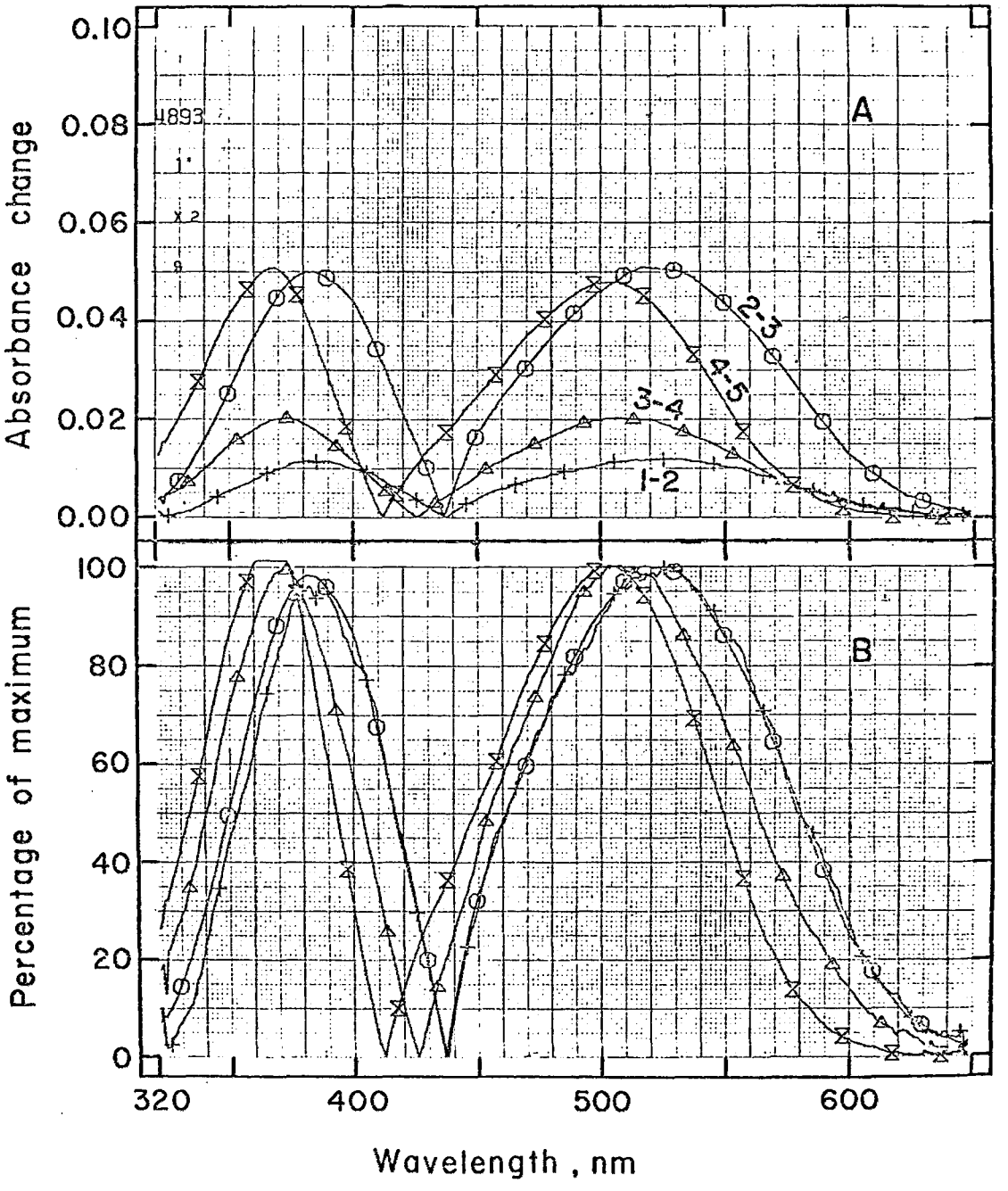

Absorbance change
0.10
0.08
0.06
0.04
0.02
0.00
A
2-3
4-5
3-4
1-2
Percentage of maximum
100
80
60
40
20
0
B
320
400
500
600
Wavelength , nm

Table 5.4. Estimates of porphyropsin λ_{max}. See text.

Experiment number	Molar % porphyropsin in extract[A]	Absorbance at λ_{max}, total extract	$\lambda_{max} \pm$ S.E., bleach 1	Absorbance at λ_{max}	$\lambda_{max} \pm$ S.E., bleach 2	Absorbance at λ_{max}	Corrected $\lambda_{max} \pm$ S.E., bleach 2
4890	64.6	0.072	523.6 ± 0.47	0.008	522.1 ± 0.21	0.031	524.0 ± 0.16
4892	65.0	0.099	523.3 ± 0.25	0.010	521.7 ± 0.25	0.043	524.0 ± 0.12
4893	61.3	0.128	523.3 ± 0.40	0.012	522.4 ± 0.38	0.051	525.6 ± 0.22
4894	87.3	0.032	527.0 ± 0.42	0.005	524.2 ± 0.18	0.018	525.3 ± 0.20
4895	99.7	0.072	527.5 ± 0.30	0.014	525.1 ± 0.20	0.047	525.3 ± 0.19
4896	80.4	0.071	523.2 ± 0.35	0.008	521.1 ± 0.23	0.038	521.9 ± 0.17
4898	77.2	0.053	526.4 ± 0.60	0.007	522.5 ± 0.26	0.030	523.7 ± 0.18
4899	80.9	0.056	525.4 ± 0.28	0.007	522.7 ± 0.13	0.028	523.8 ± 0.21
4900	73.5	0.055	525.3 ± 0.90	0.007	523.0 ± 0.22	0.025	525.0 ± 0.23
		Means:	525.0 ± 0.6 (n=9)		522.8 ± 0.4 (n=9)		524.3 ± 0.4 (n=9)

[A] calculated after λ_{max}'s had been determined.

The following three interpretations were considered: 1) a third, more red-sensitive pigment, perhaps a cone pigment, was present in the extracts in trace amounts (see Munz and McFarland, 1975). Most or all of this pigment was destroyed in the first bleach (λ680), causing the λ_{max} estimates from the first difference spectrum to be high. 2) The second bleach (λ675) bleaches porphyropsin plus a small but significant amount of rhodopsin, causing the λ_{max} estimates from the second difference spectrum to be low. 3) Both (1) and (2) are true, so that the true λ_{max} is between the two estimates.

To determine the correct interpretation, a variation of a technique used by Dartnall et al. (1961) was employed. The percentage of rhodopsin bleached by 4 min of λ675 was determined from two additional experiments (not given) to be about 10% of the initial amount (9.5 and 10.6%). The amount of rhodopsin remaining in the extract after the first bleach (λ680) was estimated from the product of bleaching curve of the difference spectrum (curve 2 - final curve)--see Allen et al. (1973). Then an absorbance spectrum of $P499.5_1$, scaled to equal 10% of this amount, was subtracted from the second difference spectrum (2-3); and the λ_{max} of the porphyropsin was determined from this new, "corrected" difference spectrum in the usual way (Table 5.4, column 8). The overall average of these λ_{max} estimates was 524.3 ± 0.4 nm, slightly but not significantly less than the overall average of the λ_{max} estimates from the first bleach, which was 525.0 ± 0.6 nm. Thus if there is a third pigment present, it is in very small amounts. I believe that as an estimate of λ_{max} 524.3 is better than 525.0 nm, since the standard errors of the individual means are smaller (compare

columns 8 and 4 of Table 5.4, and compare difference spectra 1-2 and 2-3 of Figure 4B), a simple consequence of the greater amount of pigment. Also, this estimate is less likely to be influenced by a trace amount of a third pigment.

Thus sticklebacks have the pigment pair $P499_1$-$P524_2$ (to the nearest nanometer). The difference between the λ_{max}'s of this pair is somewhat larger than usually found (see Fig. 4 of Munz and Schwanzara, 1967, and Fig. 13 of Bridges, 1972). For example, a rhodopsin with a λ_{max} of 499.4 nm would correspond to a porphyropsin with a λ_{max} near 520.7 nm, according to the empirical equation derived by Munz and Schwanzara (1967).

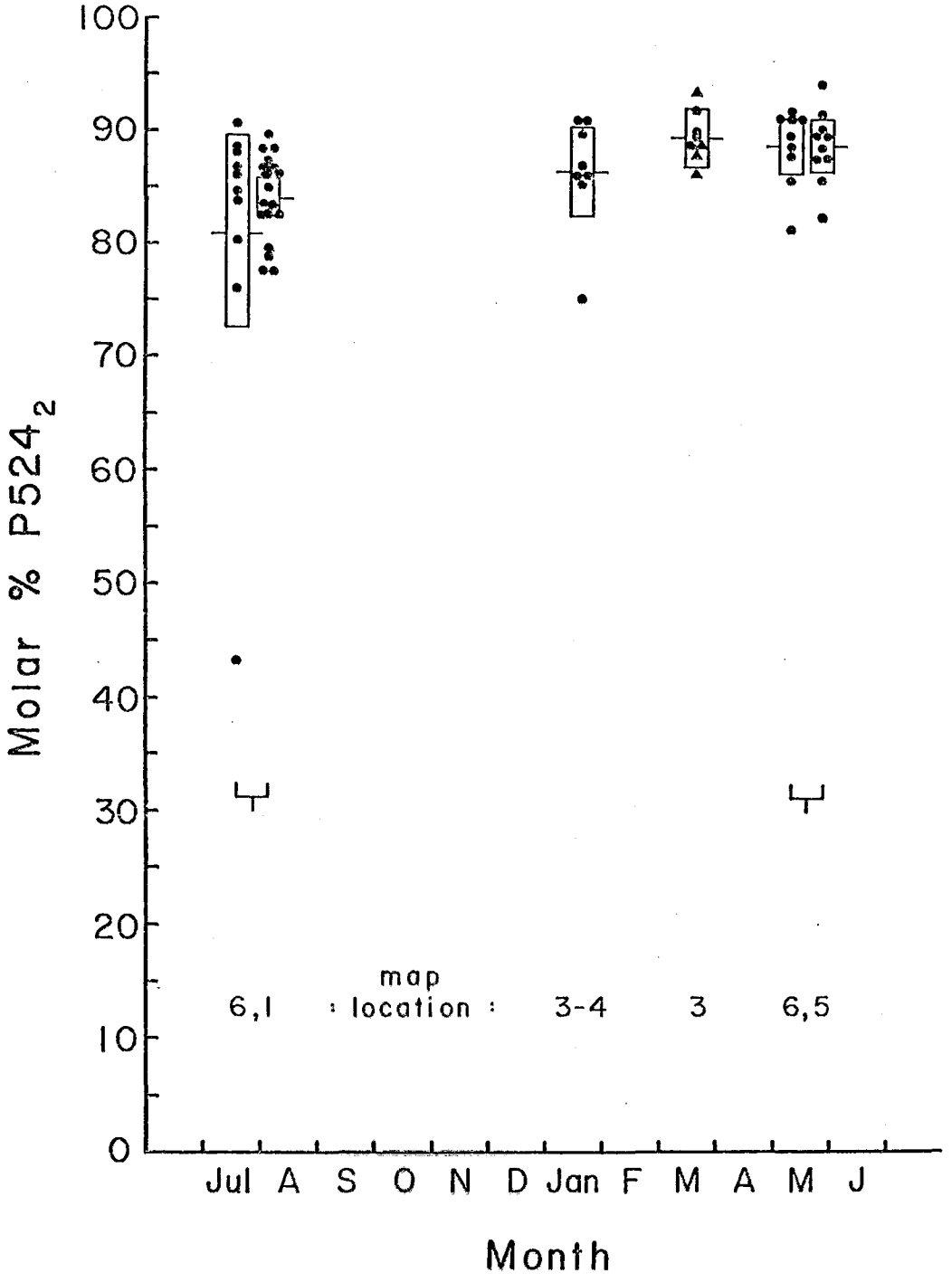
For these λ_{max} determinations, visual pigment extracts from the pure trachurus population of the Rogue River were used. The absorbance maxima of the pigment pair of the Coos Bay fish were not determined as definitively, but they are almost certainly the same: for 28 extracts, each rich in porphyropsin, the initial bleach of 2-3 min with λ680 yielded an overall λ_{max} estimate of 524.6 $\pm$ 0.4 nm for the porphyropsin.

Visual Pigments--Proportions of Rhodopsin and Porphyropsin

Coos Bay

The percentage of porphyropsin ($\%P524_2$ or %P524) in the retinas of sticklebacks captured in the Isthmus Slough area at various times of the year is given in Figure 5.5. The retinas are uniformly rich in porphyropsin, with means between 80 and 90% P524, and individuals between 75 and 95% P524, with one exception--one of the fish from the July

Figure 5.5.--Percentages of porphyropsin in sticklebacks from the Coos Bay mixed population. Circles represent extracts made from the 2 retinas of individual fish, and triangles represent extracts made from the 4 retinas of 2 fish. Horizontal lines give the means, and vertical bars give the 95% confidence limits of the means. The map locations refer to Figure 5.1. All of the fish were collected between July 1973 and May 1974, except the January group, which was collected in 1975.


100
90
80
70
60
50
40
30
20
10
0
Molar % P524$_2$
map
6,1 : location : 3-4 3 6,5
Jul A S O N D Jan F M A M J
Month

collection at location 6 had only 43% porphyropsin.

The location of the mixed population in winter is undetermined. Nine juveniles were collected in January in Isthmus Slough; the percentages of P524 of 8 of these are given in Figure 5.5. However, the fish were not plentiful--that was the total catch of half a day's effort. The fish were abundant in mid-March in Isthmus Slough, but they had not yet moved into the stream at location 6. The fish were sought around docks in the more saline parts of Coos Bay in winter, but none were found. Unfortunately, equipment for trawling the eel grass beds in winter was not available.

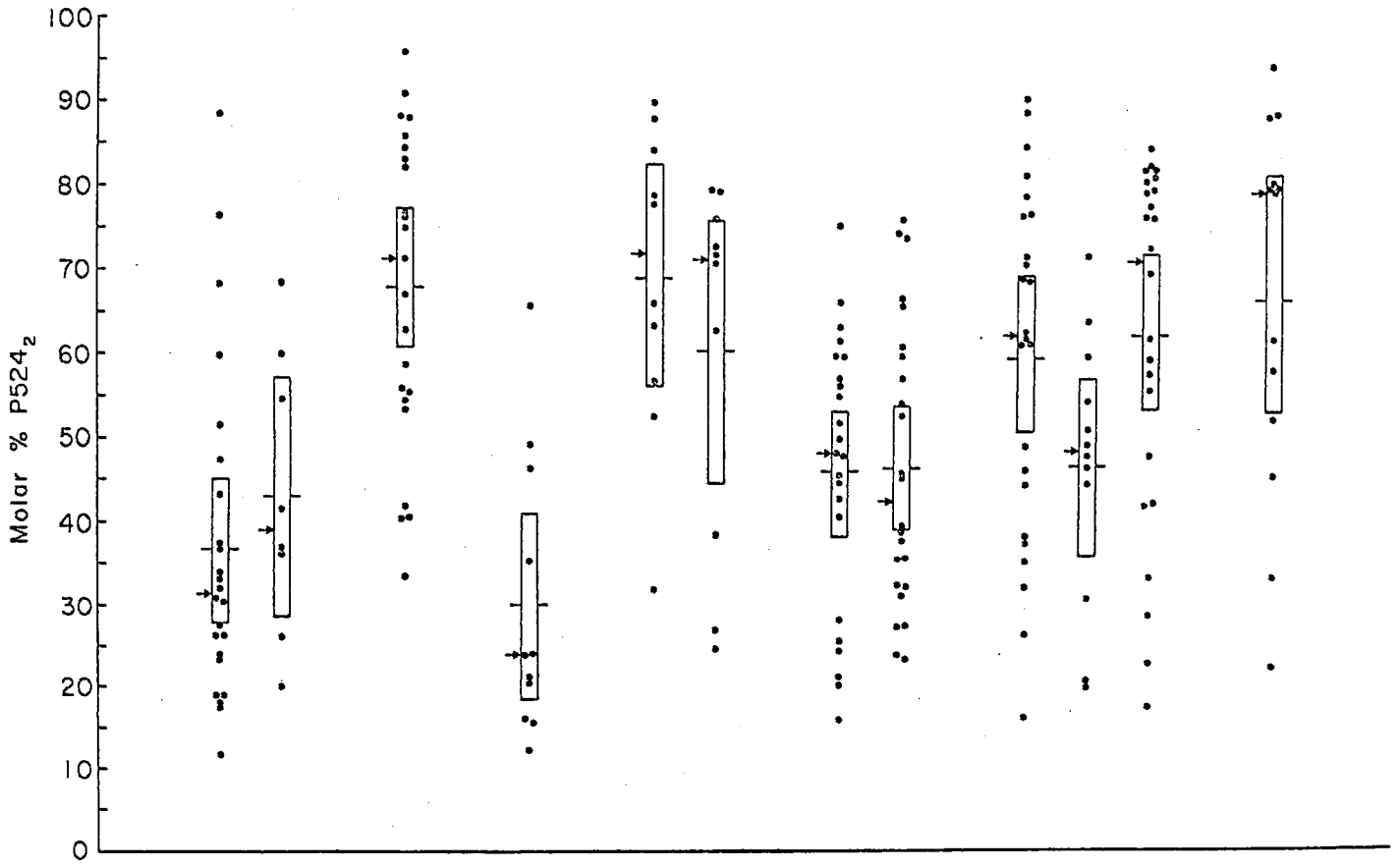
Thus it cannot be declared that the visual pigments do not change, except for the individuals which remain in Isthmus Slough. The main part of the population in winter is presumably in the more saline parts of Coos Bay or in the ocean, but the visual pigment composition of these fish has not been determined.

Rogue River

According to data kindly supplied by Ron Boyce and Steve Cramer of the Oregon Wildlife Commission, which seined the lower Rogue year round during 1974 and 1975, anadromous sticklebacks arrive in large numbers in April. These fish are all trachurus.

Visual pigment data for fish caught from mid-April till mid-August are shown in Figure 5.6. The most striking feature of the data is the great variability of the proportions of rhodopsin and porphyropsin in each of the groups. This variability will be discussed later (see Discussion). For the moment I will consider only trends in

Figure 5.6.--Percentages of porphyropsin in sticklebacks from the Rogue River pure trachurus population. Circles represent extracts from individual fish. Horizontal lines give the means, and vertical bars give the 95% confidence limits of the means. Arrows indicate the medians. The data are arranged according to calendar date, and data from 1974 and 1975 are interdigitated. For each group the date of capture, distance upstream, habitat, and relative abundance are given at the bottom. The relative abundance refers to the number of fish caught in one haul with a 100-foot beach seine: <20 fish is scored as "not abundant," >200 fish is scored as "abundant," and numbers between 20 and 200 are scored as "intermediate." The two groups marked with an asterisk were caught by trap. For explanation of "order," see text. Compare the variability within groups with that of the Coos Bay Fish, shown in Figure 5.5.



Date :	18 Apr 75		3 May 75	12 May 75	19 May 74		10 Jun 75		31 Jul 75			16 Aug 74
R.M. :	3.5	11.5	15	0.5	1.5	10	15	27	10	19-22	25	10
Habitat :	A	A	A	A	B	B	A	A	B	A	B	B
Abundant ? :	*	*	yes	no	no	yes	no	intermediate	yes	no	yes	no
Order :	2	2	3	1	4	4	5	5	6B	6A	6B	7

the means and medians (below).

The fish were caught at various distances from the mouth of the river, given at the bottom of Figure 5.6. Two types of habitat are distinguished: "A" denotes areas in the main stem of the Rogue with rocky bottoms and little vegetation; "B" denotes typical spawning sites, which are slough areas, often at the mouths of sluggish tributaries, with mud or sand bottoms and more abundant vegetation (see Figure 5.6). The relative abundance of the fish at each location and date is also given (see figure legend for explanation).

The fish caught from 18 April to 12 May 1975 in "A" sites had no trace of sexual coloration, and individual eggs in the ovaries were just distinguishable to the unaided eye. The spawning site at R. M. 10 was seined on 18 April, but the fish had not arrived there yet, although fish were trapped farther upstream in an "A" site on that date. The fish were abundant there on 19 May (previous season); a faint pink coloration was visible anterioventrally in about half of the males. Only "A" sites were examined on 10 June; the fish collected there were similar in appearance to the 19 May fish. On 31 July, fish were collected in both "A" and "B" sites. In the "B" sites, males were colored a brilliant red-to-orange, females were gravid, and juveniles were present. A total of 50-60 adults were caught in 4 "A" sites--all of these were gravid females. Juveniles were also present, but no adult males. The spawning site at R. M. 10 was seined on 16 August (previous season), but only 16 adult fish, most in poor condition, were found.

A partial interpretation of the visual pigment data, considering only trends in the means and medians, is outlined in the row labelled "Order" at the bottom of Figure 5.6. The ordering is based on date, miles upstream, and habitat as measures of how far along the fish are into the spawning migration. Thus the 12 May group at R. M. 0.5 was placed before the 18 April and 3 May groups farther upriver, because the former fish were later migrants just entering the river. The percentage of porphyropsin increases as the fish enter the river and move to the spawning sites, and it remains approximately constant thereafter (1,2,3,4,6B, and 7, where "B" refers to habitat "B").

The fish found in "A" sites when most of the fish are already in spawning sites are clearly different from the latter in their visual pigment composition (5 and 6A vs. 4, 6B, and 7). Whether this difference is due to subtle environmental differences affecting the visual pigment proportions directly or whether there is a correlation between the visual pigment composition and the final destination of the fish cannot be determined from these data. Excluding these fish (5 and 6A), the median increases from 24% P524 for fish just entering the river to 60-80% P524 for fish at the spawning sites. For the fish in "A" sites during the spawning season, the medians were 40-50% P524. (The median is a better statistic for comparison than the mean for these distributions, many of which are strongly skewed.) Note that the visual pigment change is complete before male sexual coloration is visible.

There was no significant difference between males and females, tested by two-way analysis of variance. There may be a difference between the sexes in the 18 April collection at R. M. 3.5, however. The

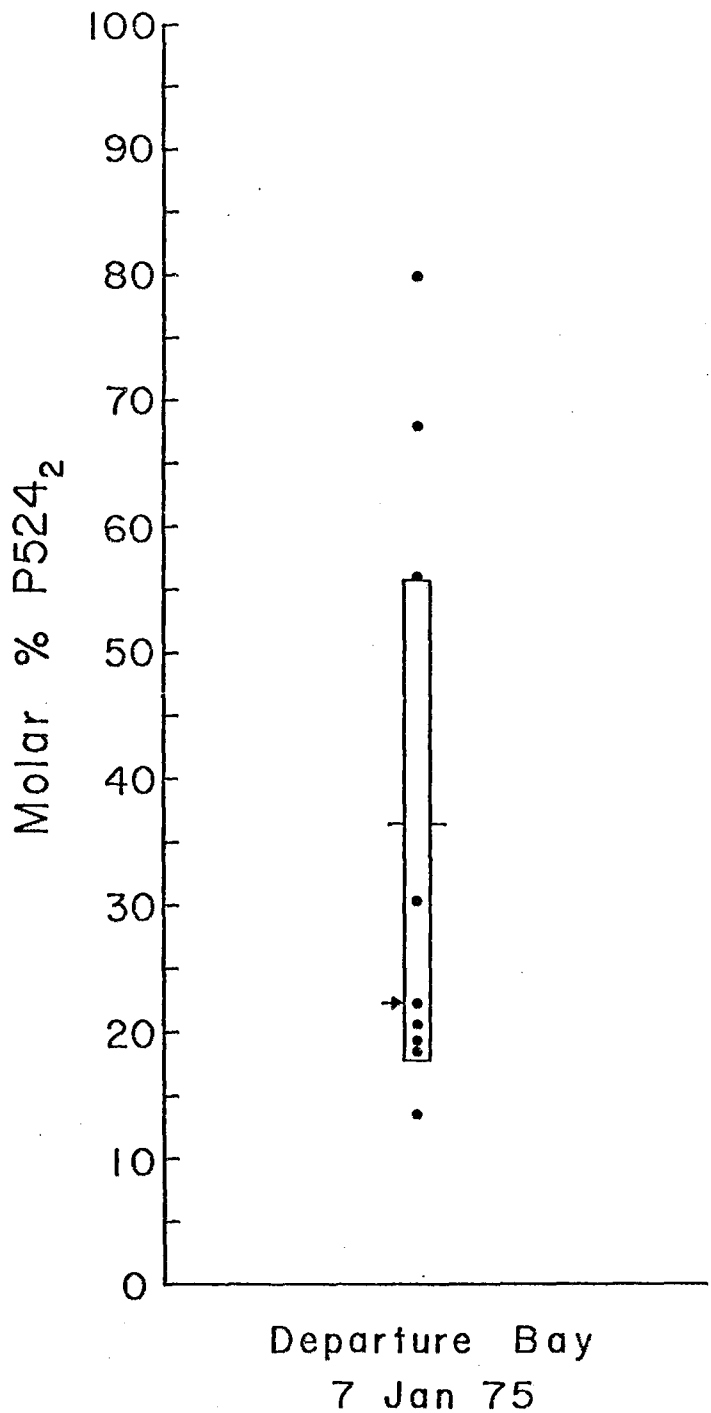
males had 21.6 ± 2.9% porphyropsin (n=6), and females had 42.8 ± 5.3% porphyropsin (n=16), on the average (the sexes of two fish were not determined). The difference arises because all 9 of the individuals with percentages of P524 greater than the overall mean were females (see Figure 5.6). There was no correlation between length and %P524. Indeed there was not much variability in the length--the fish are probably all (or nearly all) one year old, according to data on an Alaskan trachurus population by Narver (1969, quoting a MS).

British Columbia

Juvenile sticklebacks were caught at depth in Departure Bay in January, 1975, and sacrificed the evening after capture. Good pigment extracts were obtained from 9 of the 10 fish caught (Figure 5.7). The standard length of these fish varied from 2.5 to 4.2 cm, and there was an apparent correlation between size and %P524 (not shown), smaller fish having more porphyropsin (more recent arrivals from the spawning sites?). However, the number of fish examined was too small to allow a strong conclusion.

This visual pigment distribution is very similar to that of the Rogue River fish when just entering the river (Figure 5.6, 12 May 1975). However, the appropriateness of this comparison is uncertain, for the Departure Bay fish may or may not be from a pure trachurus population (see Morphology, above), and the possibility of a size correlation in the juveniles exists. More data, especially from late winter to test whether the variance decreases before the spring migration, are required.

Figure 5.7.--Percentages of porphyropsin in sticklebacks caught in Departure Bay, British Columbia, in January 1975. The type of population is uncertain (see Morphology section). Circles represent extracts from individual fish. The horizontal line indicates the mean; the vertical bar, the 95% confidence limits of the mean; and the arrow, the median. Compare this distribution with the distribution in the fish just entering the Rogue River, Oregon (Figure 6, 12 May 75), but also see text.


100
90
80
70
60
50
40
30
20
10
0
Molar % P524$_2$
Departure Bay
7 Jan 75

Juveniles from a mixed population were collected in shallower areas in Horseshoe Bay. Five visual pigment extracts were made, each using 10 fish. The %P524 varied from 39 to 48%, but the reliability of these values is uncertain because the fish were kept in darkness for 3 days before sacrifice. Constant darkness can alter the rhodopsin/porphyropsin ratio in other species, with the direction of change depending on the species (see Allen et al., 1973).

Discussion

The original purpose of these experiments was to compare the visual pigment changes in anadromous sticklebacks with those in anadromous salmonids. A full comparison can not yet be made, however, because of the uncertainty of the visual pigment proportions in sticklebacks in the sea, especially for the Coos Bay mixed population.

Rogue River

In the pure trachurus population of the Rogue River, the changes in the percentages of P524 were monitored from the time the fish were just entering the river until late in the spawning season. There was a definite trend of increasing percentage of P524 during the migration, similar to that in Pacific salmon (Beatty, 1966). However, at the spawning sites the variance was still large, whereas in salmon at the spawning sites the retinas are rich in porphyropsin (salmon have the pigment pair $P503_1$-$P527_2$) and the variance is small. During the migration the variance may be large in Pacific salmon, for example in fish caught in tidewater, but there is a strong correlation with

development of spawning colors (Beatty, 1966). In the sticklebacks, on the other hand, the visual pigment proportions have changed before the breeding colors are even faintly visible. (This does not necessarily preclude involvement of sex hormones in the visual pigment change, of course, since thresholds of response could be different--breeding colors begin to develop shortly after the visual pigment change.) Immature Pacific salmon caught in the ocean have high proportions of $P503_1$, and the variance is small (Beatty, 1966). The distribution for sticklebacks in the ocean is uncertain, but they may be variable there also (but with the median of the distribution at high values of $P499_1$) if the fish caught in Departure Bay, British Columbia, are representative (but see comments in Results section).

Frankly, the large variance in the %P524 at the spawning sites is difficult to interpret with the information now available. Bridges (1964b, 1965b) speculated that the individual variation he saw in Notemigonus, Belonesox, Hybopsis, and Mugil could broaden the spectral sensitivity of a school of fish and thus be advantageous. This idea has not been tested experimentally, but it would not apply to male sticklebacks in the spawning areas, which are strongly territorial (the individual variation occurs in both males and females at the spawning sites).

Is the spectral distribution of the light in the spawning habitats broad (either at one time, or in variations from day to day), so that this variability is "tolerated" or advantageous to the species? This question cannot be answered yet, for light measurements, during twilight when these rod visual pigments would be used, have not been

made. Studies such as the landmark work of Munz and McFarland (1973), who, working with tropical marine fishes, correlated the λ_{max}'s of the rhodopsins with the spectral distribution of the light measured in the animals' habitats during twilight and information on visual behavior, need to be extended to paired pigment species in notoriously variable freshwater habitats.

Are the differences between the Rogue sticklebacks and Pacific salmon due to differences in the time of year of the spawning migration? In most of the species of Oncorhynchus studied by Beatty (1966), the spawning migration occurs in the autumn, when the rivers are typically swollen and turbid; but the visual pigment transition also occurs in spring-run chinook salmon (O. tshawytscha). When the sticklebacks enter the Rogue in the spring, the river is also swollen and turbid, but the water becomes less turbid during the course of the spawning season (this observation was made visually and from turbidity data of the Oregon Wildlife Commission). The spawning behavior is also quite different; for example, sticklebacks breed several times during the season, whereas the salmon breed only once and die. Also of interest is the exception among the Pacific salmon: the sockeye, O. nerka, has an average of only 32% $P527_2$ at the spawning site (Beatty, 1966). The sockeye is also exceptional in that it migrates upriver in summer, when the water is less turbid, and spawns later in clear blue streams or lakes. The unravelling of all these puzzles must await further experimentation, however. Badly needed are good data on spectral distribution of light, especially at twilight, information on visual behavior, and data to determine whether the prosthetic groups of the cone pigments change in

parallel with those of the rod pigments.

Coos Bay

The migratory mixed population of Coos Bay was not sampled as extensively as the trachurus population from the Rogue River. However, the visual pigment distributions were clearly different in these two populations during the spawning season. The mean %P524 in sticklebacks from Coos Bay was higher and the variances were much smaller. The probable reason for this difference is that the water in the Isthmus Slough area of Coos Bay is turbid and stained a deep brown all year, except in the freshwater streams at locations 2 and 6 (Figure 5.1), which tend to be clearer in summer. In such habitats the light distribution should be strongly skewed to the red end of the spectrum, no matter what the incident radiation is. The fish at location 6 had similar proportions of P524, with the exception of one fish (Figure 5.5).

British Columbia

Only a small number of specimens from a population of uncertain morphology, taken in early winter, were examined (Figure 5.7). On the average, the fish had more rhodopsin than porphyropsin, but the range was large. There was a suggestion of a correlation between %P524 and size, unproved because of the small sample size. The appropriateness of comparing the visual pigments in these fish with either of the above two populations is uncertain (see Results).

Visual Pigment Proportions in "Trachurus" Sticklebacks

In the introduction I suggested that the physiology of trachurus

morphs from mixed populations might differ in detail from that of trachurus morphs from homogeneous populations. That certainly seems to be true for visual pigment proportions (compare Figures 5.5 and 5.6). During the spawning season trachurus morphs from the Coos Bay mixed population had high mean values of %P524, with little variability within groups, whereas the trachurus from the homogeneous population from the Rogue River had somewhat lower mean values, and with considerable variability. Moreover, the porphyropsin percentages did not differ among the morph types in the mixed population. In preliminary experiments (unpublished), treatments which changed the mean percentages of P524 had little or no effect on the inherent variability or lack of variability in the two populations. Therefore the suggestion, that closer attention be given to the type of population from which trachurus morphs are selected for physiology experiments, is reinforced.

CHAPTER VI

PRELIMINARY EXPERIMENTS ON THE CONTROL OF VISUAL PIGMENT PROPORTIONS IN STICKLEBACKS

This chapter describes a series of preliminary experiments with sticklebacks from either the mixed population from Isthmus Slough (Coos Bay) or the pure trachurus population of the Rogue River. Effects of light intensity, temperature, salinity, and thyroxine have been tested. These experiments are considered preliminary because of problems with design or with mortality, which will be discussed in detail.

Materials and Methods

Sticklebacks (Gasterosteus aculeatus) from either the mixed population in Isthmus Slough (Coos Bay) or the pure trachurus population in the Rogue River were used in each experiment. The fish were adults and of both sexes. Data on the dates the fish were caught, the locations caught, and the salinity and temperature at each site are compiled in Table 6.1.

Irradiance measurements were made and the λP_{25}, λP_{50}, and λP_{75} points were calculated as described in Chapter III.

Fish were fed lightly with frozen brine shrimp in all experiments except #4, in which they were not fed.

Mortality was high in some experiments and differential among groups in some. Because this may affect conclusions, mortality information is given for each experiment. When mortality is given as a

Table 6.1. Catch data for fish used in the experiments.

Experiment number	Site of capture	Date caught	Salinity	Temperature
1	Isthmus Slough, location 1	27 Jul 73	7‰ at low tide	19.5 °C
2	same	same	same	same
3	Rogue River, R.M. 10	19 May 74	fresh water	13.5
4	Rogue River, R.M. 3.5 and 11.5	18 Apr 75	edge of tidewater and fresh water	9
5	Rogue River, R.M. 15	3 May 75	fresh water	10

fraction, the denominator gives the actual number of fish at the start of the experiment, i.e., the fraction is not reduced.

Mixed Population

Fish captured in the brackish waters of Isthmus Slough on 27 July 1973 were used in Experiments 1 and 2. The collection comprised 38% leiurus, 28% semiarmatus, and 34% trachurus (see Table 5.1 of preceding chapter). No distinction among morph types was made in assigning fish to groups.

Experiment 1

The fish were divided into 6 groups on 29 July. Three groups were kept in an experimental coldroom maintained at 12 $\pm$ 1° C and with a "short day" of 8:16 light:dark. The other 3 groups were kept on a table in my office with a "natural long day" via a large window; the water temperature was controlled at 20 $\pm$ 2° C by circulating water through glass coils in the aquaria.

The fish in the coldroom were illuminated from above with cool-white fluorescent lamps. The light intensity was 6.39×10^{14} photons $\cdot cm^{-2} \cdot sec^{-1}$ [410-700 nm], and the λP_{25}, λP_{50}, and λP_{75} points were 536, 573, and 604 nm, respectively. The fish in the office were illuminated with cool-white lamps from above as well as with variable sunlight through the window. The contribution from the lamps was 9.69×10^{14} photons$\cdot cm^{-2} \cdot sec^{-1}$ [410-700 nm], and the spectral distribution was identical to that above; the contribution from sunlight was not measured.

In each room one group was kept in fresh water (dechlorinated tap water), one group was kept in one-third sea water, and one group was kept in one-third sea water in which 1 ppm L-thyroxine (the sodium heptahydrate salt; Nutritional Biochemicals) was dissolved. Fish were kept in 17-liter glass aquaria, and water was replaced every 4-5 days.

The fish were sacrificed after 29-30 days (20° groups) or 35-37 days (12° groups).

Mortality for the groups is given in the order in which they appear in Figure 6.1, starting with the second from the left (i.e., omitting the initial group): 7/25, 15/26, 11/28, 21/23, 15/25, and 10/24.

Experiment 2

The fish used in this experiment had been kept for 99 days in one-third sea water in the coldroom described above. The temperature, photoperiod, and light intensity during this time and during the experiment were the same as described for the coldroom in Experiment 1.

The fish were divided into 3 groups: one group was transferred to fresh water (dechlorinated tap water), one group was transferred to sea water, and the third group was retained in one-third sea water. Fish were kept in 17-liter glass aquaria, and water was replaced weekly.

The fish were sacrificed after 72-81 days.

Mortality was low: 3/15, 1/14, and 1/15 for the fresh water, one-third sea water, and sea water groups, respectively.

Pure Trachurus Population

Fish captured in the Rogue River were used for Experiments 3, 4, and 5 (Table 6.1). All were trachurus morphs.

Experiment 3

Fish were caught at the spawning site at river mile 10 on 19 May 1974. About half of the males had faint pink color (beginning of breeding colors).

Fish were kept in fresh water for 15 days in the same coldroom used above. The fish were divided into 2 groups: one group was retained in fresh water, and the other group was transferred to one-third sea water for 8 days and then to sea water. After this group had been held in sea water for 42 days, the remaining 8 fish of the freshwater group (FW_1) and 8 of the 19 remaining fish in the sea water group (SW_1) were sacrificed. The mortality was different in the 2 groups: 15/23 and 4/23 for the fresh water and sea water groups, respectively. The 11 remaining fish in sea water were kept another 40 days and then sacrificed (SW_2); 5 of the 11 died.

The temperature during the experiment was 12 $\pm$ 1° C. The photoperiod was initially 9:15 light:dark, but it was changed to 8:16 when the one group was transferred from one-third sea water to sea water. The light intensity was the same as in Experiments 1 and 2. Fish were kept in 17-liter glass aquaria.

The fresh water used was dechlorinated tap water to which the following concentrations of salts were added: 0.09 mM NaCl, 0.01 mM KCl, 0.08 mM $MgSO_4$, and 0.30 mM $CaCO_3$. The $CaCO_3$ was largely insoluble,

however, which may account for the high mortality in the freshwater group. In the following experiments (#4 and #5) the completely soluble $CaCl_2$ was used. According to Smith (1962, M.Sc. thesis, University of British Columbia), the addition of $CaCl_2$ at a concentration of 0.5 mM "permitted 100% survival of anadromous fish [sticklebacks] while the calcium concentration does not exceed that found naturally in many British Columbia streams and lakes. This was required because normally anadromous Gasterosteus aculeatus show a very high mortality when placed in dechlorinated Vancouver tap water with its low ion content (Smith, 1962 [this refers to his B.A. thesis, also at U.B.C.])."

Experiment 4

Fish were caught at river miles 3.5 and 11.5 on 18 April 1975. The fish were quite silvery and showed no traces of spawning colors.

Fish were kept in one-third sea water in a 400-liter galvanized horse trough ("stock tank"), painted with white Tygon paint, until the experiment started. One half of the trough was covered with a sheet of blue plexiglass (#2424, 0.125 inches thick), and the other half was covered with plywood. The fish congregated under the plywood. The trough was in the coldroom used in the experiments above--hence the illumination was light from the cool-white lamps passing through the blue plexiglass. The downwelling irradiation, measured under the plexiglass, was 8.16×10^{13} photons$\cdot$cm$^{-2}\cdot$sec^{-1} [410-700 nm], and the λP_{25}, λP_{50}, and λP_{75} values were 453, 474, and 492 nm, respectively. Under the plywood and facing the bright end of the tank, the (horizontal) irradiation was 5.78×10^{12} photons$\cdot$cm$^{-2}\cdot$sec^{-1} [410-700 nm], and the

spectral distribution was similar.

After 15 days the fish were divided into 5 groups: one group was dark-adapted and sacrificed (initial group), one group was put in constant darkness (in one-third sea water), and 3 groups were transferred to 17-liter glass aquaria and illuminated by the cool-white lamps at an intensity of 1.79×10^{15} photons$\cdot cm^{-2} \cdot sec^{-1}$ [410-700 nm]--this intensity was (intentionally) greater than that in the experiments above, because the aquaria were put on shelves rather than on the floor. Of the 3 illuminated groups, one was kept in sea water, one in one-third sea water, and one in fresh water (dechlorinated tap water plus the following concentrations of salts: 1.5 mM $CaCl_2$, 0.4 mM NaCl, 0.05 mM KCl, and 0.5 mM Na_2HPO_4). The fish were sacrificed after 6 weeks in these conditions.

The temperature was 11 $\pm$ 1° C and the photoperiod was 12:12 light:dark during the 15 days before the experiment and during the experiment.

There was no mortality in any of the illuminated groups. There was some mortality in the constant darkness group when the air supply was accidentally blocked, but none thereafter (this group was checked at night with a dim red safelight).

Experiment 5

Fish were caught at river mile 15 on 3 May 1975. The fish were quite silvery and showed no traces of spawning colors.

Fish were kept in 2 horse troughs (outfitted as described for Experiment 4) for 50-52 days before the experiment began. The

temperature was 11 $\pm$ 1° C, the photoperiod was 12:12 light:dark, and the illumination was "blue" as described above.

Fish taken from the stock tanks were divided into 12 groups, one of which was dark-adapted and sacrificed immediately (initial group). The remaining groups were kept in various combinations of temperature, illumination, and salinity, which are given in Table 6.2. Fish were sacrificed at various intervals up to 4 weeks. Because of the large number of fish in this experiment, the start and finish of the experiment were staggered over 3 days. Thus the pre-experiment adaptation in the horse troughs varied (trivially) from 50 to 52 days, depending on the group, but the lengths of time in the experimental conditions were the same for all groups.

The following mortality figures are given in terms of per cent mortality per month, for comparative purposes. The original stock comprised approximately 1200 fish. The mortality during the 50 days before the experiment began was 6%/mo. The mortality during the experiment rose to 21%/mo. during the first 2 weeks and to 47%/mo. during the last 2 weeks. The mortality was roughly uniform among the groups, except that the mortality was lower in the freshwater groups. The mortality in the stock tanks also rose sharply during the experiment--it was 62%/mo. during the last 2 weeks of the experiment. The reason for this sudden increase in mortality is unclear--it may have been due to an epidemic of disease, or it may have been due to sudden release of sex hormones into the blood (sticklebacks are less euryhaline during the spawning season). The latter explanation seems more probable,

since there were no overt signs of disease and the mortality was lower in the freshwater groups.

Visual Pigment Analysis

Fish were dark-adapted at least 2 hours before sacrifice. The 2 retinas of individual fish were pooled for each extract in Experiments 1, 2, and 3. The 4 retinas of pairs of fish were pooled for each extract in Experiments 4 and 5. The methods of preparing and analyzing stickleback visual pigments are given in the preceding chapter.

Statistical Analysis

Statistical analyses were performed as in the preceding chapter.

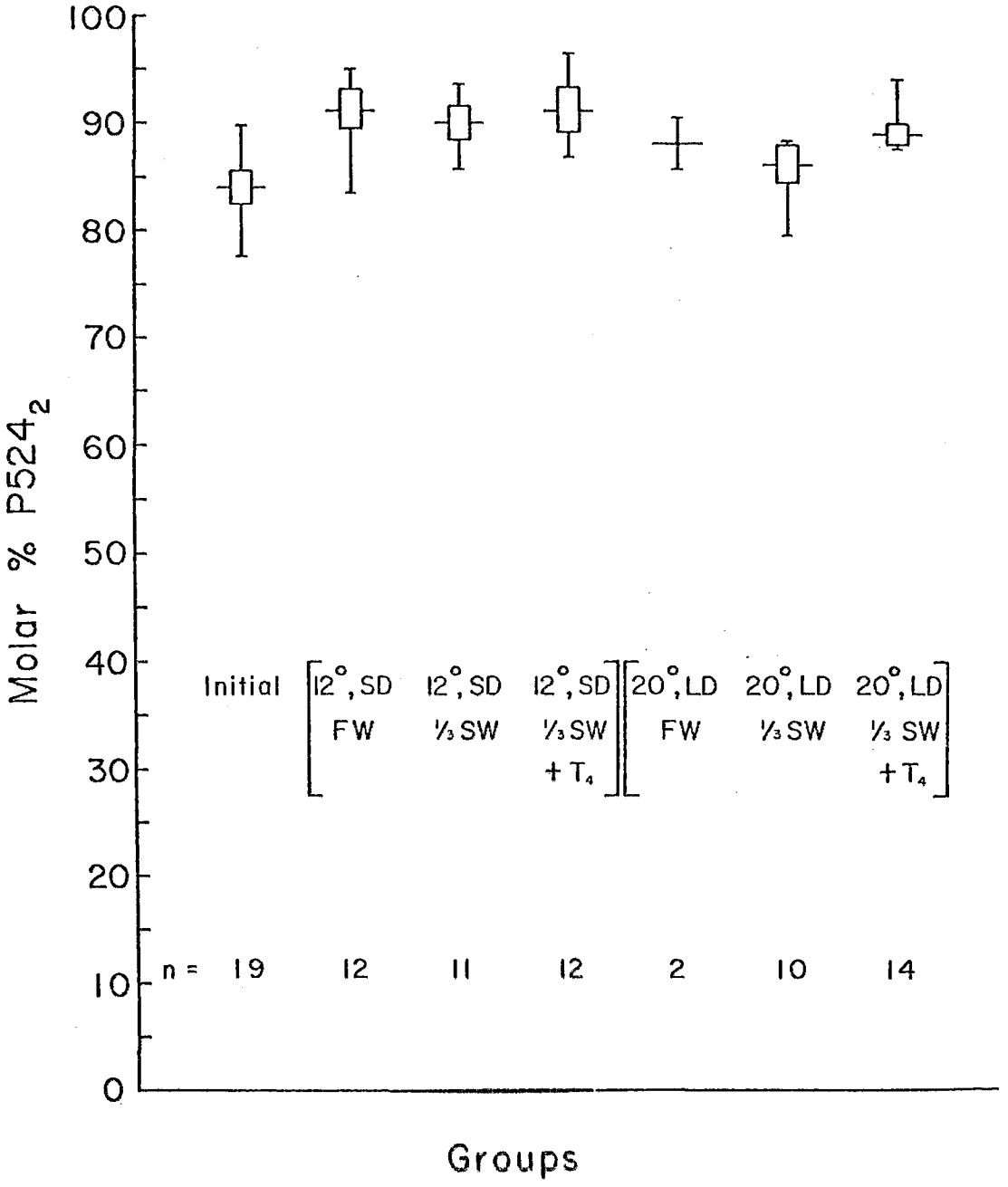
Results

Population

Experiment 1

The fish, when caught in late July, had high percentages of porphyropsin in their retinas (mean = 84%; initial group, Figure 6.1). Experiment 1 was an attempt to induce "winter levels" of porphyropsin, postulated to be low by analogy with juvenile Pacific salmon caught in sea water (Beatty, 1966). Fish were kept in fresh water, in one-third sea water, or in one-third sea water plus 1 ppm thyroxine in each of two rooms--one room was maintained at 12° C and had a "short day" of 8:16 light:dark with artificial "white" light, and the other room was warmer (aquaria water maintained at 20° C) and a "natural long day"

Figure 6.1.--Experiment 1. The horizontal lines give the means, the vertical bars give the 95% confidence limits of the means, and the vertical lines indicate the ranges. The group mnemonics are defined as follows: SD = short day; LD = long day; FW = fresh water, 1/3 SW = one-third sea water; 1/3 SW + T_4 = one-third sea water, in which 1 ppm thyroxine is dissolved. See text for description of experiment.


100
90
80
70
60
50
40
30
20
10
0
Molar % P524$_2$
Initial
12°, SD FW
12°, SD ⅓ SW
12°, SD ⅓ SW + T$_4$
20°, LD FW
20°, LD ⅓ SW
20°, LD ⅓ SW + T$_4$
n = 19 12 11 12 2 10 14
Groups

(sunlight through a large window during August, supplemented with artificial "white" light). Fish were kept in these conditions for approximately one month.

The results are shown in Figure 6.1. A two-way analysis of variance was performed (the initial group was excluded for this analysis). The difference between rooms was highly significant ($p < .001$), and the difference among groups within rooms was significant at a low level ($p < .05$). However, the groups under a short daylength at 12° were sacrificed nearly a week after the groups under a long daylength at 20°, so that the comparison between rooms is not valid. More important, all the groups have increased in %P524--none of the 12°, short day groups decreased as postulated. Moreover, the differences among groups within each room are very small. There was a significant difference because of a consistent pattern in both rooms (FW and 1/3SW+T_4 groups each higher than the 1/3SW group, as predicted on the basis of thyroxine effects in other fishes and the postulated fresh water-sea water difference in migratory sticklebacks) and the small variances within groups, but the differences are too small to be very meaningful.

Experiment 2

This experiment was done to determine whether full strength sea water and/or a longer period of time in saline water at a short daylength were required for the %P524 to decrease. A small stock of the sticklebacks collected in July had been kept for 99 days in one-third sea water at short daylength in the coldroom. At the start of the experiment groups were transferred to fresh water, one-third sea water,

or sea water, and kept for 72-81 days in these conditions before sacrifice. The results are shown in Figure 6.2. There was no significant difference among the groups, and all 3 groups were high in P524.

Pure Trachurus Populations

Experiment 3

Fish captured in mid-May 1974 at a spawning site had an average of 60% P524, and the variance was large (Figure 6.3). Fish were kept in fresh water or sea water for 6 weeks at 12° C and an 8:16 light:dark cycle ("white" light). The %P524 in the freshwater group increased and the variance decreased, whereas the opposite was true for the sea water group (groups FW_1 and SW_1, Figure 6.3). The difference between the two means was significant ($p < .05$) using a special t-test for heterogeneous variances (Sokal and Rohlf, 1969). Fish kept another 6 weeks in sea water (SW_2) were similar in %P524 to the SW_1 group (Figure 6.3).

The interpretation is confounded by the much larger mortality in the freshwater group (see Materials and Methods). There could be a correlation between visual pigment composition and ability to survive in fresh water of low calcium content, or they could be a direct influence of salinity. In the following experiments $CaCl_2$ was added to the fresh water.

Experiment 4

Fish caught in mid-April 1975 at 3.5 and 11.5 miles upriver (and not yet at spawning sites) had mean percentages of P524 of 37% and 43%,

Figure 6.2.--Experiment 2. Symbols and mnemonics are as described in legend of Figure 6.1. See text.

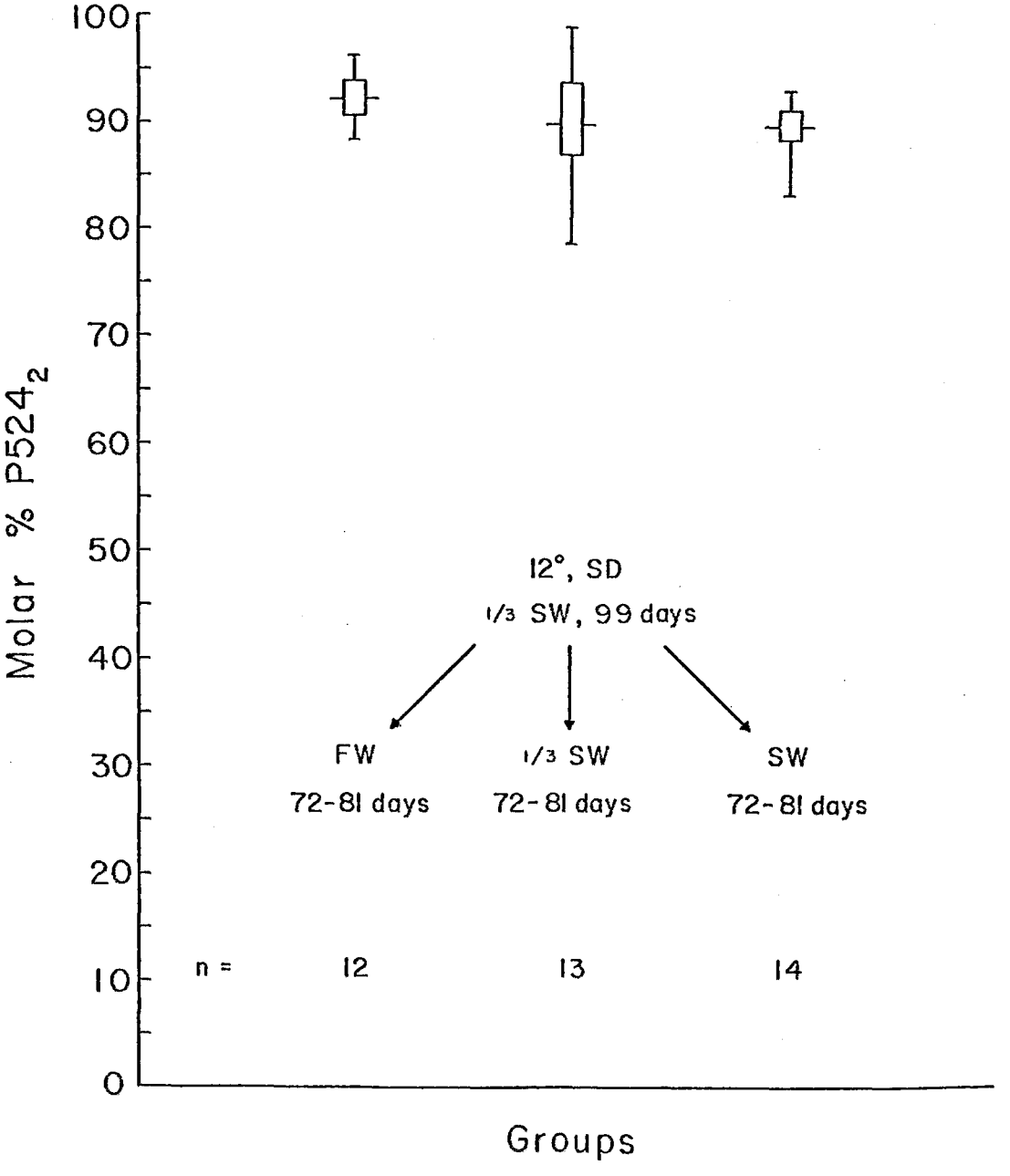
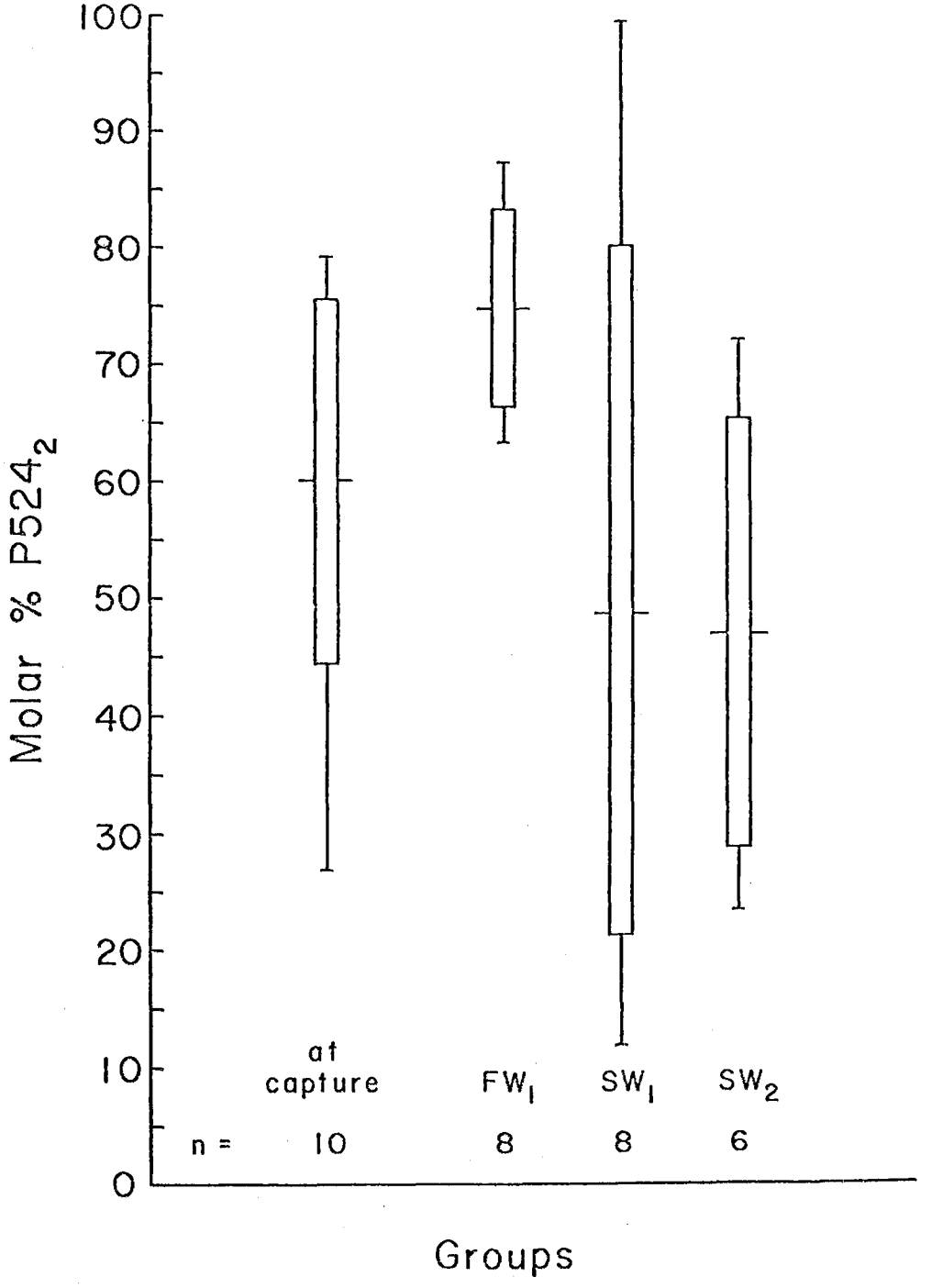

100
90
80
70
60
50
40
30
20
10
0
Molar % P524$_2$
12°, SD
1/3 SW, 99 days
FW
72-81 days
1/3 SW
72-81 days
SW
72-81 days
n =
12
13
14
Groups

Figure 6.3.--Experiment 3. Groups FW_1 and SW_1 were kept in fresh water and sea water, respectively, for 42 days. Group SW_2 was kept in sea water an additional 40 days. See text.


100
90
80
70
60
50
40
30
20
10
0
Molar % P524$_2$
at capture
FW$_1$
SW$_1$
SW$_2$
n =
10
8
8
6
Groups

respectively (Figure 5.6 of preceding chapter). Fish from these two collections were pooled for this experiment.

The mean %P524 in fish kept for 15 days in one-third sea water in dim "blue" light with a 12:12 photoperiod and at 11° C decreased slightly but not significantly from that of the above groups (to 35%--initial group, Figure 6.4). Fish were then transferred to "white" light, also with a 12:12 photoperiod, and kept in fresh water, one-third sea water, or sea water, or they were transferred to constant darkness and kept in one-third sea water. The results are shown in Figure 6.4. The difference between the constant dark group and the "white" light groups was significant at $p < .01$. There was also a significant difference among the 3 salinities ("white" light groups) at $p < .05$, but the regression against salinity was not significant. However, to demonstrate a significant relationship between %P524 and salinity would require large sample sizes, because the variances within groups remain large. (Note: the sample sizes given at the bottom of Figure 6.4 indicate the numbers of extracts in each group, and retinas from 2 fish were used to make each extract in this experiment and in the following experiment. When this factor is considered, the variances are similar to those in Figure 5.6 of the preceding chapter.)

Experiment 5

Fish caught in early May 1975 at river mile 15 (and not yet at spawning sites) had mean percentages of P524 of 68% (Figure 5.6 of preceding chapter). When the fish were kept for 50 days in one-third sea water in dim "blue" light with a 12:12 photoperiod at 11° C, the

%P524 decreased to 24% (initial group, Figure 6.5), near the value when the fish were just entering the river.

Fish were then transferred to various combinations of temperature, light intensity, and salinity, and fish from each group were sacrificed after 2 and 4 weeks (and fish from the (T14,I2,1/3SW) group were also sacrificed after 1 week). The results are shown in Figures 6.5, 6.6, and 6.7, and the group mnemonics are explained in Table 6.2. The results of statistical tests are given in Table 6.3.

Temperature had no clear effect (Figure 6.5). Although at 4 weeks the linear regression was significant ($p < .05$), the analysis of variance was not significant (one cannot put much faith in a significant regression when the differences among the groups are not significant).

Light intensity did have an effect, higher intensities favoring porphyropsin (Figure 6.6). The differences among groups were significant at both 2 and 4 weeks, and the linear regression was significant at 2 weeks. A difficulty is that the means of the groups appeared to be increasing after 2 weeks (see also the I2 group at 1 week), but the means were lower at 4 weeks! The reason for this anomaly may lie in the increasing mortality during this experiment: the fish with higher proportions of porphyropsin may have been less capable of surviving in one-third sea water.

Salinity had no clear effect, either in groups illuminated with "blue" light or held in constant darkness. The difference among groups was significant ($p < .01$) in one case, but there was no clear relationship with salinity (see Table 6.3 and Figure 6.7).

Figure 6.4.--Experiment 4. See text.

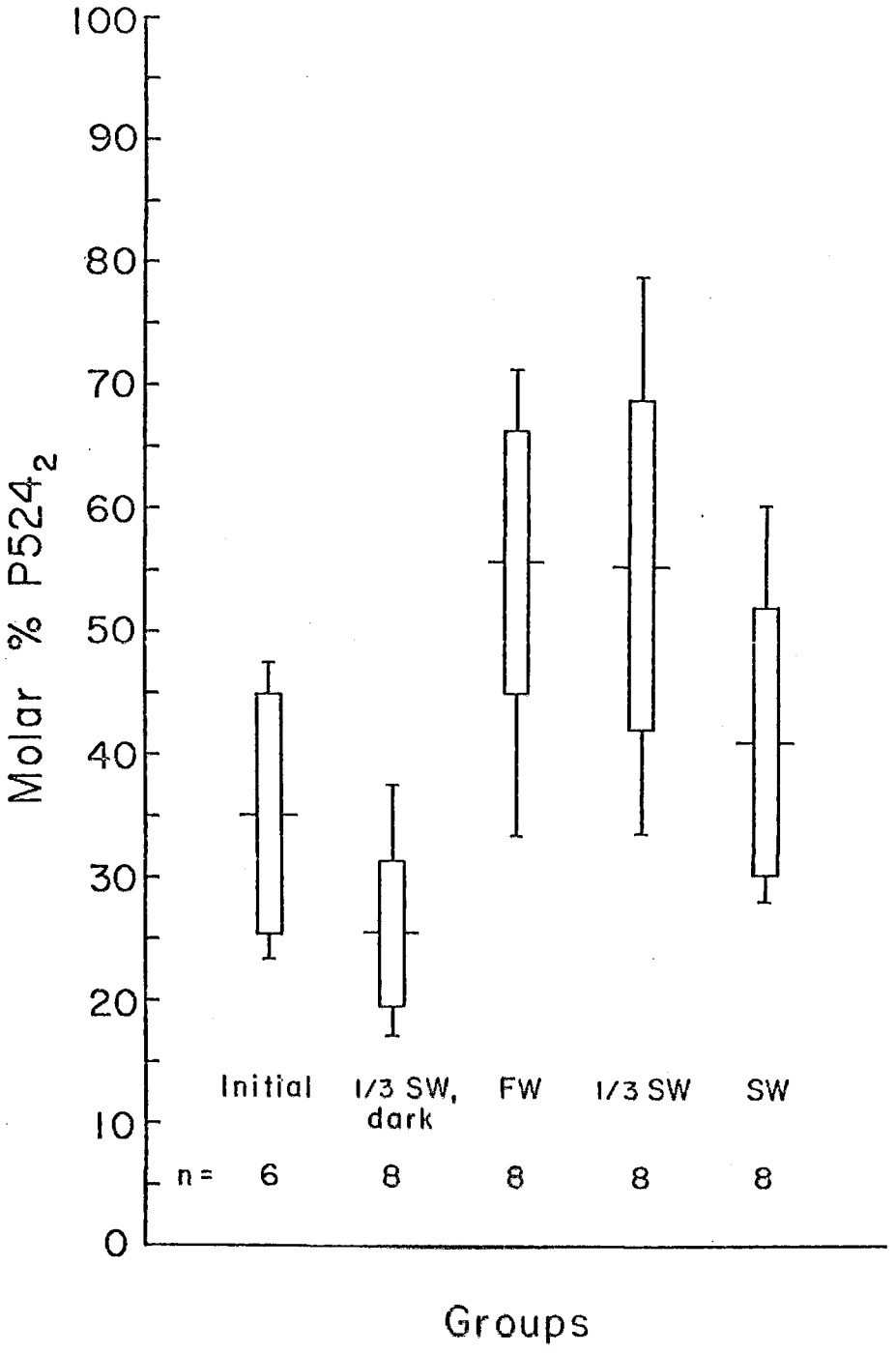

100
90
80
70
60
50
40
30
20
10
0
Molar % P524$_2$
Initial
1/3 SW, dark
FW
1/3 SW
SW
n = 6 8 8 8 8
Groups

Figure 6.5.--Experiment 5. Effects of temperature. Group mnemonics are explained in Table 6.2. The group marked with an asterisk is the same group similarly marked in Figures 6.6 and 6.7. Results of significance tests are given in Table 6.3. See text.

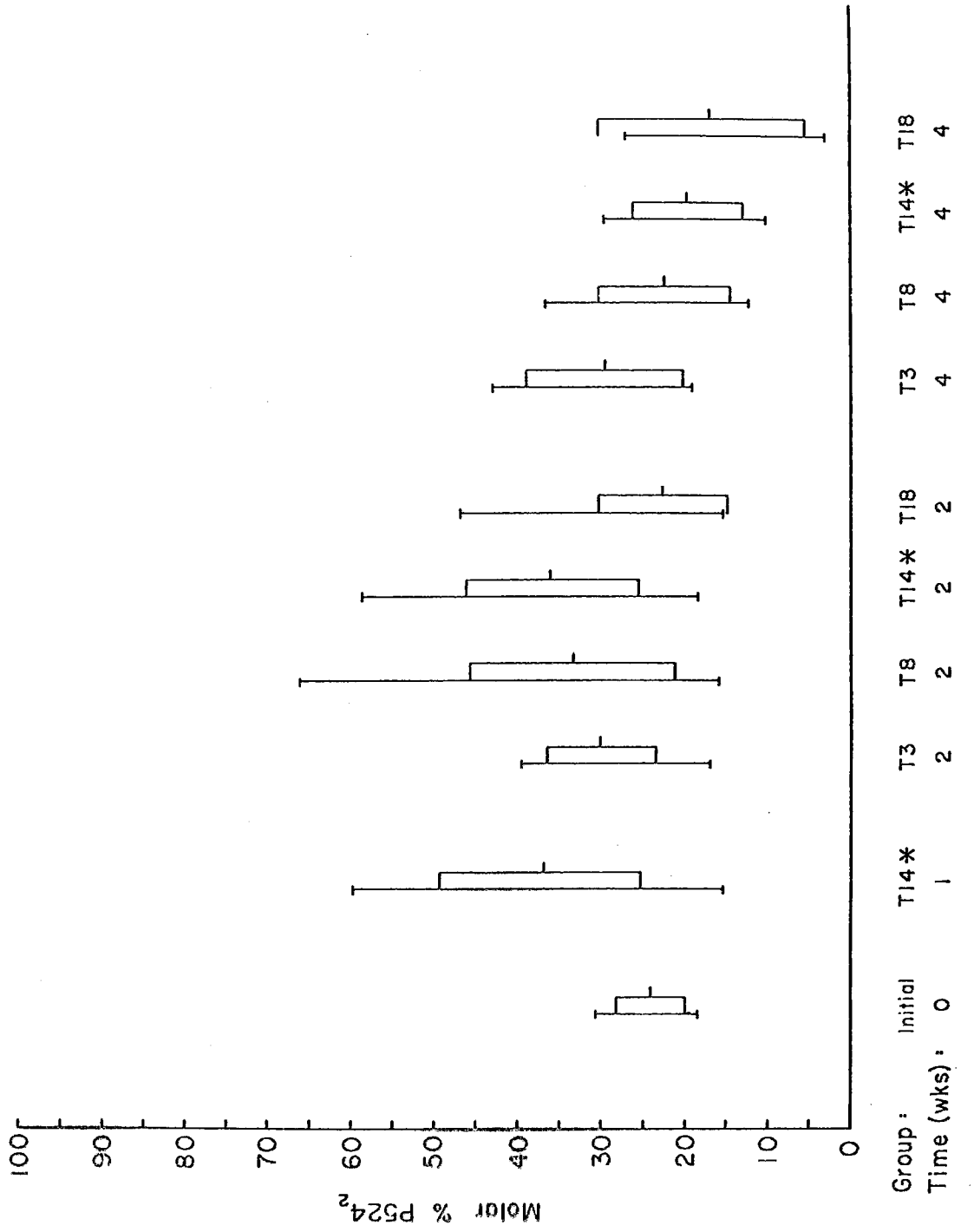

Molar % P524
100
90
80
70
60
50
40
30
20
10
0
Group: Initial T14* T3 T8 T14* T18 T3 T8 T14* T18
Time (wks): 0 1 2 2 2 2 4 4 4 4

Figure 6.6.--Experiment 5. Effects of light intensity. See Tables 6.2 and 6.3 and text.

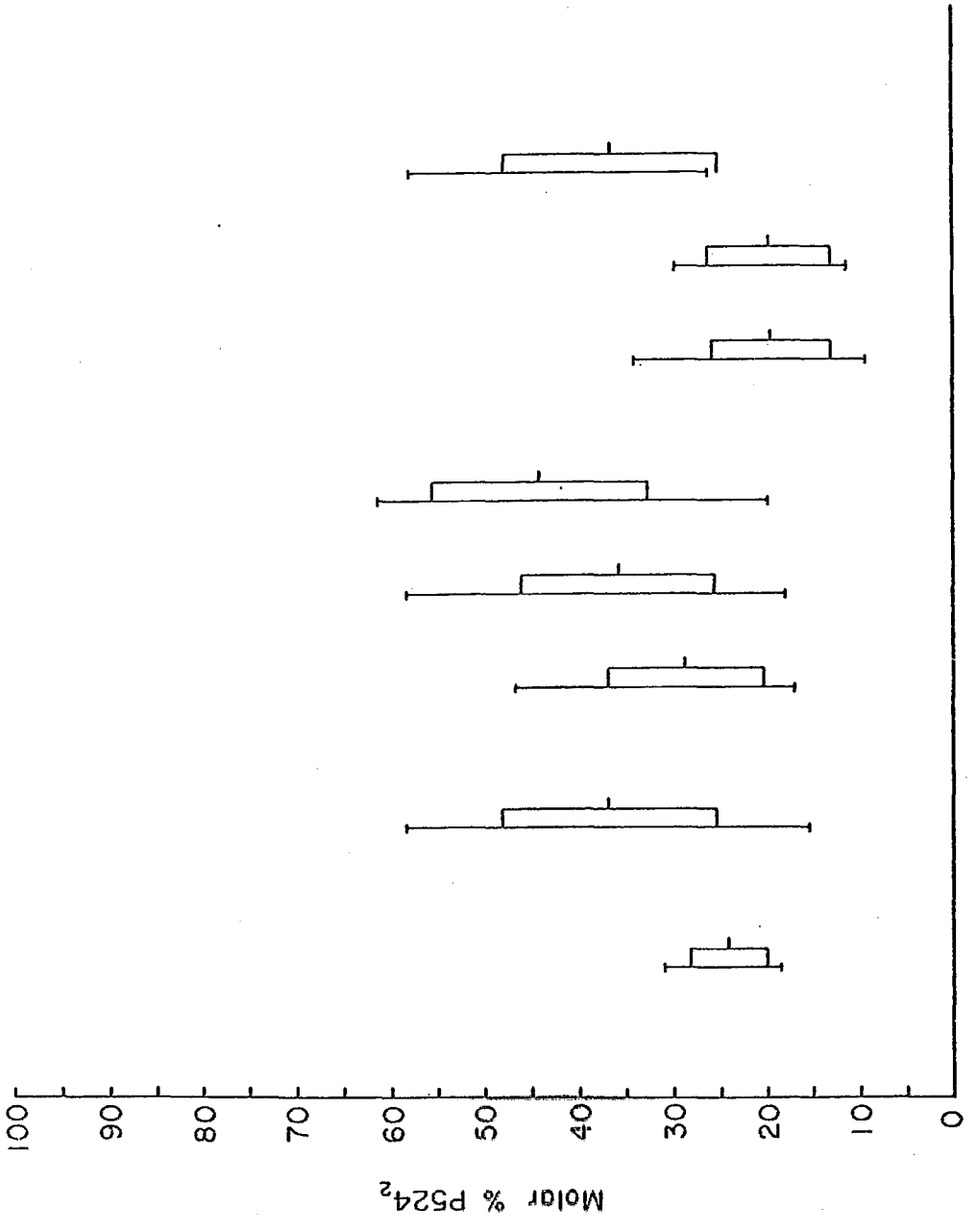

Molar % P524
100
90
80
70
60
50
40
30
20
10
0
Group: Initial I2* I1 I2* I3 I1 I2* I3
Time (wks): 0 1 2 2 2 4 4 4

Figure 6.7.--Experiment 5. Effects of salinity. See Tables 6.2 and 6.3 and text.

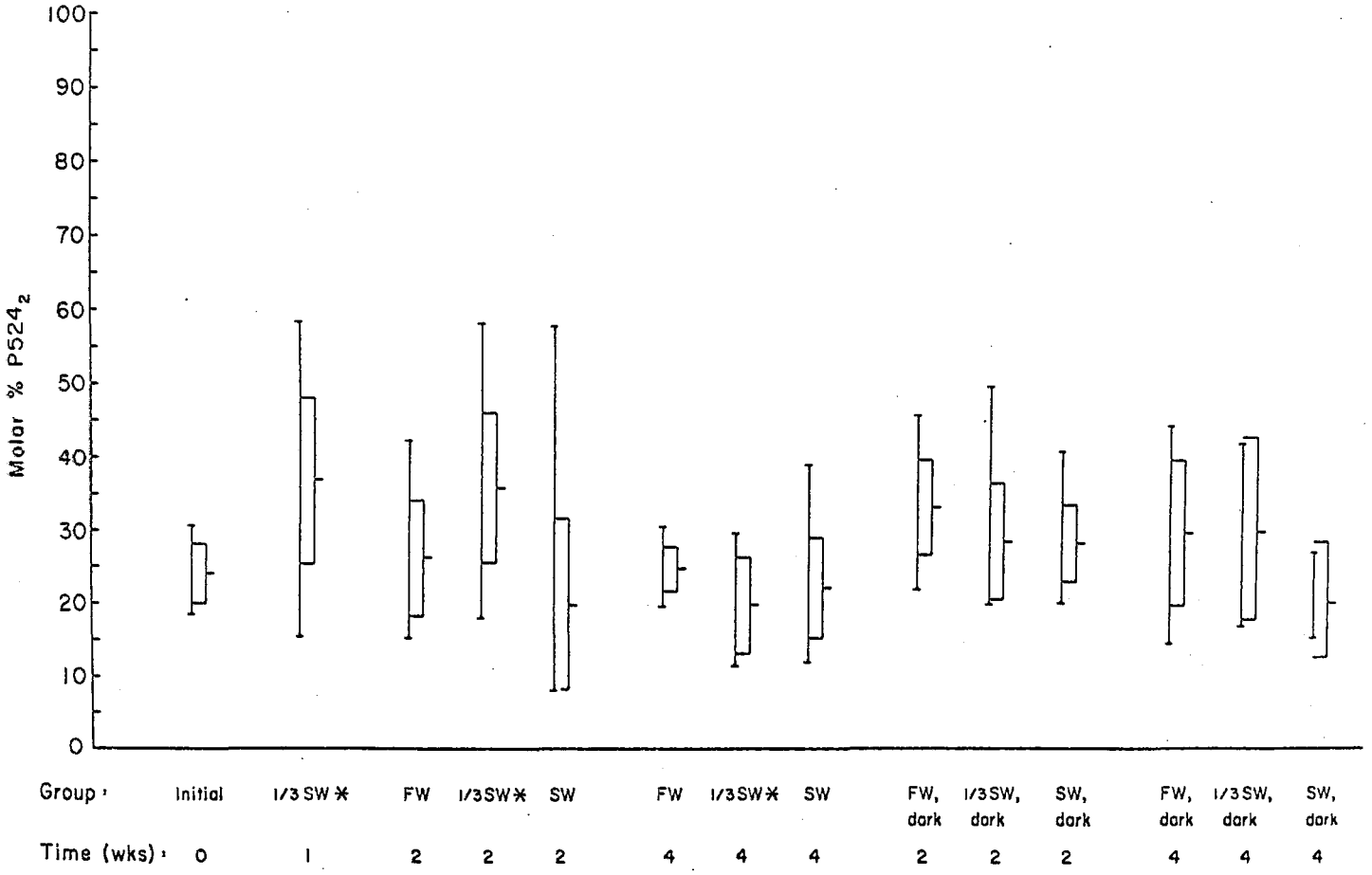

Molar % P524$_2$
100
90
80
70
60
50
40
30
20
10
0
Group: Initial 1/3 SW * FW 1/3 SW * SW FW 1/3 SW * SW FW, dark 1/3 SW, dark SW, dark FW, dark 1/3 SW, dark SW, dark
Time (wks): 0 1 2 2 2 4 4 4 2 2 2 4 4 4

Table 6.2. Details of experimental conditions for Experiment 5. Fish were kept in the experimental coldroom described in Chapter III. The illumination was "blue," and the photoperiod was 12:12. Water temperatures were controlled as described in Chapter III. The group mnemonics marked with an asterisk all refer to the same group.

Group mnemonic	Temp. (°C)	Light intensity in photons·cm^{-2}·sec^{-1} [410 - 700 nm]	Water	Number of extracts[C]	
				at 2 weeks	at 4 weeks
T3	3	1.77×10^{13}	one-third sea water	8	6
T8	8	"	"	8	7
T14*	13 - 15[A]	"	"	8	8
T18	18	"	"	8	5
I1	13 - 15[A]	5.91×10^{11}	one-third sea water	8	8
I2*	"	1.77×10^{13}	"	8	8
I3	"	5.85×10^{14}	"	8	7
FW	13 - 15[A]	1.77×10^{13}	fresh water[B]	8	8
1/3SW*	"	"	one-third sea water	8	8
SW	"	"	sea water	8	8
FW, dark	14 - 16[A]	constant darkness	fresh water[B]	8	7
1/3SW,dark	"	"	one-third sea water	8	5
SW, dark	"	"	sea water	8	4

[A] The water temperature rose during the experiment, because the room refrigeration could not cope with a summer heat wave. The constant dark groups, kept in enclosed, aerated milk cans, were warmer because of reduced evaporation.

[B] Calcium chloride was added to dechlorinated tap water at a concentration of 0.5 mM.

[C] The retinae of 2 fish were pooled for each extract. Sample sizes for the initial group and the (T14, I2, 1/3SW) group at one week were 8 each.

Table 6.3. Significance tests for Experiment 5. The levels of significance are indicated as ns (not significant), * (significant at $p < .05$), or ** (significant at $p < .01$).

Test parameter	Time	Significance among groups	Significance of linear regression
temperature	2 weeks	ns	ns
	4	ns	*
light intensity	2	*	*
	4	**	ns
salinity (illuminated groups)	2	**	ns
	4	ns	ns
salinity (dark groups)	2	ns	ns
	4	ns	ns

The striking feature of these data (Figures 6.5, 6.6, and 6.7) is that the means are all relatively low--between 15% and 45% P524. (Recall that the fish had an average of 68% porphyropsin in their retinas when collected.) Also, the variances tend to be large.

Discussion

Mixed Population

For Experiments 1 and 2, in which the percentages of P524 were high in all groups, I offer three alternative interpretations: 1) The retinas are rich in porphyropsin all year, and there is no mechanism for changing the proportions to high values of rhodopsin. In the preceding chapter fish were captured from March till July in Isthmus Slough, and some juveniles were captured there, atypically, in January. All were high in %P524. However, fish were not captured in more saline areas in the winter, disallowing a conclusion. If the fish are in Coos Bay proper, which is turbid and colored green to yellow during the winter, the fish might be expected to have porphyropsin-dominated retinas, since longer-wavelength light should dominate in such waters (see Munz, 1965). 2) The illumination was unnaturally bright, forcing the visual pigment proportions to high values of %P524. Bridges (1972) reported that some tropical freshwater fishes kept in unnaturally bright aquaria have mostly rhodopsin, whereas fish of the same species may have appreciable proportions of porphyropsin in the wild. Since light and darkness can have opposite effects in different species (Allen et al., 1973), the reverse situation could be occurring here. (The results of Experiments 4 and 5 with the Rogue sticklebacks are consistent

with this hypothesis.) Unfortunately, similar experiments were not done at lower light intensities. 3) Migratory sticklebacks may live only one year (mixed population in Europe--van Mullem and van der Vlugt, 1964; pure trachurus population in Alaska--Narver, 1969, quoting a 1966 MS), so that these experiments, using adults collected near the end of the spawning season, may be completely worthless. The experiments may be worth repeating with juveniles or with adults just entering the slough in the spring and using less intense light, should a natural seasonal-migratory change be found.

Pure Trachurus Population

Adults were also used in these experiments, but they were collected prior to spawning, when the visual pigment proportions might be more capable of change. This ability to change is best seen in Experiment 5, in which the trend of increasing %P524 during the migration (previous chapter) was reversed by the conditions in the stock tanks. The fish used in Experiments 4 and 5 had not begun to develop spawning colors, and the fish used in Experiment 3 had only faint traces of breeding coloration.

The clearest result was that higher intensities of light favored higher percentages of P524 (Figure 6.6, Table 6.3). The results of Experiment 4 (Figure 6.4) are consistent with this result--the groups kept in bright white light had higher percentages of P524 than the group in constant darkness or the initial group. These results are similar to the effects of light intensity in salmonids (see Chapter III). Since Baggerman (1957) has reported a strong photoperiodic effect in

the development of breeding condition in migratory sticklebacks, the effect of photoperiod should be tested. Such an experiment must be carefully controlled, since daily quantum dosage as well as instantaneous light intensity could have an effect independent of photoperiod (see Allen, 1971).

The interpretation of the salinity experiments is problematic. In Experiment 3 (Figure 6.3), the mean %P524 in the freshwater group was significantly ($p < .05$) higher than the mean of the sea water group, but the variance of the latter group was quite large and the mortality in the freshwater group was higher. In Experiment 4 (Figure 6.4), the means of the freshwater and one-third sea water groups were higher than the mean of the sea water group. The analysis of variance was significant ($p < .05$), but linear regression was not. In Experiment 5 (Figure 6.7, Table 6.3), there were no significant differences in three of four analyses of variance; and in the one case with a significant difference among the means, there was no clear trend.

It may be worth mention that in all six of the regression tests of %P524 against salinity (Figures 6.2, 6.4, and 6.7, i.e., including the mixed population experiment), the regression coefficient was negative. Thus there may be a weak effect of salinity, since the probability of all six coefficients being negative is 1/64 if there is no effect. Beatty (1966) found no effect of salinity on the visual pigments in juvenile Pacific salmon.

All of these experiments must be considered preliminary, because of problems with design (bright lights and use of adults at end of spawning season in Experiments 1 and 2) or with mortality (Experiments 1,

3, and 5). Valuable information has been gained on how to do future experiments with sticklebacks, however, and the effect of light intensity was clear. A test of the effect of the stock tank conditions (see Experiment 5) on the visual pigments of juvenile or young adult sticklebacks from the mixed population might yield valuable insights. A major problem in experiments with the pure trachurus population is the large variances within groups. In Experiments 4 and 5, I used 16 fish per group and the retinas from two fish for each extract, in order to narrow the confidence limits of the means without making the amount of spectrophotometric work unmanageable. More fish per group and more retinas per extract may have to be used. Also, keeping the fish in one-third sea water for 50 days before Experiment 5 was too long, since mortality increased suddenly during the experiment.

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