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VISUAL PIGMENTS AND VITAMINS A IN FISHES AND AMPHIBIANS

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Visual Pigments and Vitamins A in Fishes and Amphibians," submitted by Andrew Tsang-Cheung Tsin in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Paired visual pigment fishes and amphibians possess rhodopsin and porphyropsin which are derived from the respective conjugation of retinal (the aldehyde of retinol, vitamin A_1) and 3-dehydroretinal (the aldehyde of 3-dehydroretinol, vitamin A_2) to a species specific opsin. The relative proportions of porphyropsin and rhodopsin in these paired pigment species are known to change seasonally, during spawning migrations and at metamorphosis. The composition of visual pigments can also be artificially altered by light, temperature and hormonal treatments.

By transferring rainbow trout to new photic and thermal environments and sampling at intervals, the dynamics of the changing visual pigment composition was studied. Within 30-50 days of acclimation, visual pigment composition in different groups subjected to the same treatment converged and stablized. Groups of trout held under 15 different light and temperature regimes for 60 days were then analysed for visual pigment proportions. These 'steady-state visual pigment compositions' reflect the effect of light and temperature on the visual pigment composition in trout. The liver of these animals had mostly 3-dehydroretinol and the relative proportions of the two vitamins were not affected by light and temperature treatments.

On the basis of a light and temperature effect on the photopigment composition in the retina, it was possible to identify a rhodopsin in goldfish which were thought to possess only porphyropsin. Further experiments showed that an optimal light period (16L/8D) and

a light intensity (under a 7.5W light bulb), in combination with a high water temperature (30°C), resulted in a change of visual pigments from predominately porphyropsin to predominately rhodopsin in the goldfish retina. The relative proportions of 3-dehydroretinol and retinol in the pigment epithelium also decreased when the fish were acclimated to become rhodopsin rich. However, the relatively slow rate of change in the vitamin A composition suggests that the pigment epithelium does not regulate visual pigment composition in the retina simply on the basis of the relative amounts of its retinol and 3-dehydroretinol. These fish also possessed high proportions of 3-dehydroretinol in their livers.

After subjecting groups of leopard frogs and bullfrogs to different light and temperature regimes for 30-40 days, the former had only rhodopsin with predominately retinol in their livers. The latter however, had predominately rhodopsin in some groups but up to 50% porphyropsin in others. This is because the dorsal retina in the bullfrog responded to light and temperature treatments by a change in the proportions of rhodopsin and porphyropsin while the ventral retinas remained rhodopsin rich. The vitamin A composition in the associating dorsal and ventral pigment epithelium was, in most cases, similar to the visual pigment composition in the retina. Their livers had predominately retinol irrespective of the light and temperature conditions.

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CHAPTER I

GENERAL INTRODUCTION

Rhodopsin and porphyropsin are scotopic visual pigments[1] of vertebrates and are derived from the respective conjugation of retinal (the aldehyde of retinol) and 3-dehydroretinal (the aldehyde of 3-dehydroretinol) to opsin (for review, see Knowles and Dartnall, 1977b). Whereas rhodopsin is found in all classes of vertebrates, porphyropsin has been confirmed only in certain lampreys, osteichthyan fishes and amphibians; and it may also occur in freshwater turtles (for reviews, see Crescitelli, 1972; Knowles and Dartnall, 1977e). In certain fishes and amphibians, rhodopsin and porphyropsin may co-exist in the retina (for reviews, see Bridges, 1972; Beatty, 1975a). When the rhodopsin and porphyropsin are both based on the same opsin, the animal is referred to as a paired pigment species (see Bridges, 1972; Knowles and Dartnall, 1977c).

Many paired pigment species can alter their visual pigment (or photopigment) composition (i.e. the relative amount of rhodopsin and porphyropsin usually designated as percent porphyropsin or porphyropsin proportion). In fishes, natural changes in porphyropsin ratios may occur seasonally, during a spawning migration and at sexual maturation. Winter caught fishes, for example, may have significantly higher porphyropsin ratios than summer fishes (rudd, *Scardinius erythrophthal-*

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[1] Scotopic visual pigments refer to rod pigments which possess lower visual thresholds than photopic cone visual pigments.

mus, Dartnall, Lander and Munz, 1961; golden shiner, *Notemigonus crysoleucas*, Bridges, 1964; pike killifish, *Belonesox belizanus*, Bridges, 1964; juvenile coho and king salmon, *Oncorhynchus kisutch* and *O. tshawytscha*, Beatty, 1966; burbot, *Lota lota*, Beatty, 1969a; redside shiner, *Richardsonius balteatus*, Allen, 1971; cutthroat trout *Salmo clarkii*, Allen, McFarland, Munz and Poston, 1973). Anadromous Pacific salmon, *Oncorhynchus* spp. (Beatty, 1966) and rainbow smelt, *Osmerus eperlanus* (Bridges and Delisle, 1974) shift from predominately rhodopsin in sea water to almost pure porphyropsin during their migration to their fresh water spawning sites. Catadromous eels, however, have porphyropsin-dominated retinas when in the immature yellow stage (American eel, *Anguilla rostrata*, Beatty, 1975b), whereas rhodopsin dominates the eyes of the maturing silver adults (Wald, 1960; Beatty, 1975b).

Photopigment conversion[1] induced by light treatment was first reported in the rudd by Dartnall *et. al.* (1961). Subsequent studies have shown that an increase in light intensity or longer photoperiod may either increase or decrease porphyropsin ratios, depending on the species tested. Light favors an increase in porphyropsin in the redside shiner (Allen, 1971), rainbow trout (Allen *et. al.*, 1973; Cristy, 1976; Tsin, 1976; McFarland and Allen, 1977) and brook trout, *Salvelinus fontinalis* (Allen *et. al.*, 1973; McFarland and Allen, 1977). However light promotes a decrease in porphyropsin ratios in the rudd

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[1] Visual pigment conversion or turnover refers to the replacement of rhodopsin by porphyropsin, or vice versa, resulting in a change in the relative proportions of the two pigments in the retina.

(Dartnall *et. al.*, 1961; Bridges and Yoshikami, 1970a), pike killifish (Bridges, 1965a), golden shiner (Allen and McFarland, 1973) and common shiner (*Notropis cornutus*, McFarland and Allen, 1977). Allen and McFarland (1973) were the first to report that higher temperatures favored lower porphyropsin ratios in the golden shiner. Subsequent reports on the temperature influence suggested that higher temperatures always favored lower porphyropsin proportions, irrespective of the species response to light (rainbow trout, Cristy, 1974; Tsin, 1976; Tsin and Beatty, 1977a; McFarland and Allen, 1977; common shiner, McFarland and Allen, 1977). The possibility that environmental factors may exert their influence via a neuroendocrine system was raised (Beatty, 1975a) as thyroid hormones and prolactin were found to increase effectively porphyropsin porportions in many paired pigment fishes (thyroid hormone (s): rainbow trout, Munz and Swanson, 1965; Jacquest and Beatty, 1972; Tsin and Beatty, 1977b; kokanee, *Oncorhynchus nerka*, and coho salmon, Beatty, 1969b, 1972; redside shiner, Allen, 1971; Altantic salmon, *Salmo salar*, Beatty, 1975a; common shiner and brook trout, McFarland and Allen, 1977; prolactin: rainbow trout, Cristy, 1974).

Changes in the visual pigment composition of certain amphibians also occur. During metamorphosis, a shift from porphyropsin to rhodopsin is associated with the change from an aquatic to terrestrial habitat in many species of frogs (*Hyla* and *Rana* spp., see Bridges, 1972, for reviews). Visual pigment changes induced by light treatment in tadpoles were first reported by Bridges (1970, 1974). Light, in comparison to darkness, favored higher proportions of porphyropsin in

the tadpoles of the bullfrog *(Rana catesbeiana)*, leopard frog *(Rana pipiens)* and the bronze frog *(Rana clamitans)* (Bridges, 1970, 1974). Lower water temperature may also favor more porphyropsin in bullfrog tadpoles (Bridges, 1974). Whether light and temperature can also affect visual pigments of an adult amphibian is not known at present, although adult bullfrogs in the winter were reported to possess more porphyropsin in the dorsal retina (Reuter, White and Wald, 1971). With respect to hormones, thyroxine is known to increase rhodopsin proportions in the tadpoles of the bullfrog (Wilt, 1959) and the bronze frog (Bridges, 1975) whereas prolactin, an antagonist of thryoxine, will increase porphyropsin proportions in bullfrog tadpoles and in the eastern spotted newts, *Notophthalmus viridescens* (Crim, 1975a & b).

More studies are needed to elucidate the effect of light and temperature on the visual pigment composition in paired pigment animals. For example, is the porphyropsin proportion in the retina determined by a particular light and temperature regime? If so, how long does it take to reach a stablized porphyropsin ratio (or steady-state visual pigment composition)? Are these steady-state visual pigment compositions predictable and reproducible? What are the effects of light and temperature on these steady-state porphyropsin ratios? The present study on rainbow trout (Chapter II), a species known to possess paired visual pigments (Munz and Beatty, 1965), shows that visual pigment composition will stablize after 30-50 days of acclimation. This steady-state visual pigment composition is predictable and is appropriate for the study on the effects of light and temperature. Studies on steady-state visual pigment composition, as

obtained from subjecting groups of trout for 60 days to 15 different light-temperature regimes (i.e. 6^0C, 12^0C and 18^0C in combinations with 0L, 6L, 12L, 18L and 24L), suggest that longer photoperiods and lower water temperatures always favor higher porphyropsin proportions in trout.

The subsequent chapter (Chapter III) demonstrates how light and temperature can be utilized to alter the visual pigment composition in the goldfish, *Carassius auratus*. Although goldfish were thought to possess only porphyropsin for the past decade (Schwanzara, 1967; Bridges, 1973), the present study shows that by subjecting goldfish to certain combinations of photoperiod and temperature, a detectable level of rhodopsin can be identified. Moreover, an almost complete replacement of porphyropsin by rhodopsin was achieved by employing a particular combination of light intensity, photoperiod and temperature (see Chapter III for details). Consequently, it is likely that light and temperature effects on visual pigment composition of the goldfish may have led previous investigators to believe that goldfish possess only porphyropsin.

The labile nature of visual pigments in paired pigment species is not restricted to paired pigment fishes and amphibian tadpoles only. Reuter *et. al.* (1971) reported that winter bullfrogs had more porphyropsin (about 40%), all segregated into dorsal retina, than the summer frogs (about 5% porphyropsin). They attributed this change in visual pigment composition to a seasonal effect. It would be of interest, therefore, to see whether this seasonal change of visual pigment composition is also attributable to possible effects of light and temperature, as has been suggested in paired pigment

fishes. My subsequent study on the adult bullfrog *(Rana catesbeiana)* and the adult leopard frog *(Rana pipiens)* (Chapter IV) may provide an answer to this question. My results showed that the dorsal retina of the adult bullfrog responded to different photoperiod and temperature treatments by possessing predominately rhodopsin or predominately porphyropsin (see Chapter IV) while the ventral portions of the retina remained rhodopsin rich. However, the visual pigment composition of the adult leopard frog did not respond to similar light and temperature treatments (they possessed only rhodopsin in their retinas, see Chapter IV). These results substantiated Bridges' suggestion that the adult bullfrog may constitute an exception amongst species of *Rana* and *Hyla* in retaining porphyropsin in their adult phase (Bridges, 1974). Nevertheless, this interesting phenomenon of light and temperature induced visual pigment conversion occurring only in the dorsal part of the retina, but not the ventral part, should prove to be useful for future studies on the mechanism of visual pigment turnover in the vertebrate retina.

The mechanism of visual pigment turnover is at present, a topic for investigation. In a paired pigment animal, rhodopsin and porphyropsin are respectively derived from retinal and 3-dehydroretinal conjugated to the same opsin (Knowles and Dartnall, 1977c). The replacement of rhodopsin by porphyropsin, therefore requires a change of the prosthetic group from retinal to 3-dehydroretinal. Retinal and 3-dehydroretinal in the retina come from retinol and 3-dehydroretinol provided to the retina by the adjacent pigment epithelium which stores

the two vitamins A[1] mostly in the form of retinyl or 3-dehydroretinyl esters (for reviews of the biochemistry of the visual processes, see Bridges, 1967; Morton, 1972; Bridges, 1975 and Knowles and Dartnall, 1977d). Wald (1939), Wilt (1959) and Reuter *et. al.* (1971) reported that vitamin A compositions in the bleached retina and the pigment epithelium are similar in several species (king salmon, rainbow trout and brook trout, Wald, 1939; bullfrog tadpoles, Wilt, 1959; bullfrogs, Reuter *et. al.*, 1971). Assuming the vitamin A compositions in the bleached retinas reflect the visual pigment compositions, Reuter *et. al.*, (1971) suggested that the pigment epithelium regulates the visual pigment proportions through the relative proportions of its vitamin A_1 and vitamin A_2. Bridges and Yoshikami (1970b) confirmed that porphyropsin proportions in the retina 'mirrored' the 3-dehydroretinol/retinol ratios in the (saponified) pigment epithelium of the rudd. Therefore they also suggested that the pigment epithelium controls visual pigment composition in the retina through a change in the relative availability of its retinol and 3-dehydroretinol. Moreover, when the bleached retina of the goldfish (concluded to contain only porphyropsin) was placed on *Rana pipiens* pigment epithelium (concluded to contain only vitamin A_1), it regenerated mostly rhodopsin

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[1] In this thesis, vitamin A_1 represents the combined retinol and retinyl ester whereas vitamin A_2 represents the combined 3-dehydroretinol and 3-dehydroretinyl ester. The saponified pigment epithelial extracts contain only the alcohols (retinol and 3-dehydroretinol) whereas the non-saponified pigment epithelial extracts may contain both alcohols (retinol and 3-dehydroretinol) and esters (retinyl ester and 3-dehydroretinyl esters). The term vitamin A means both vitamins.

(Bridges, 1973), confirming the influence of pigment epithelial vitamin A composition on the visual pigment composition in the retina.

The hypothesis that the carotenoid composition of the pigment epithelium determines the visual pigment composition in the retina needs to be critically re-examined because: 1) the assumption that the vitamin A composition in the bleached retinas reflects the visual pigment composition may not be valid because retinas may possess a considerable storage of vitamin A not conjugated to form visual pigments (from 0.1 moles per mole of visual pigment to 1.4 mole/mole in different species examined, Bridges, 1975). Since (Reuter *et. al.* (1971), Wald (1939) & Wilt (1959) did not prewash the dark adapted retinas with light petroleum ether before bleaching (a procedure which will remove the stored vitamin A without affecting the visual pigment, Wald, 1935-6), their unbleached preparation may have contained a significant amount of free vitamin A that was in a proportion different from that present in the visual pigments; 2) recent reports have shown that 3-dehydroretinol proportions in the pigment epithelium do not necessarily reflect the porphyropsin proportions in certain species, including bullfrog tadpoles and bronze frog tadpoles (Bridges, 1975); and 3) results from pigment regeneration studies may be inappropriate to elucidate the mechanism of visual pigment conversion. This is because pigment regeneration is different from new pigment synthesis[1]

[1] Retinal is conjugated to opsin forming rhodopsin at the base of the rod outer segment during disc renewal which occurs in the light and in the dark (in *Rana pipiens*, Hall and Bok, 1974). Under illumination, rhodopsin is bleached and during dark adaptation it is regenerated throughout the rod outer segment thus providing an additional route of retinal incorporation with opsin to form rhodopsin (Hall and Bok, 1974). It is assumed that the biosynthesis of porphyropsin from opsin and 3-dehydroretinol, in addition to the

(at the base of the rod outer segment; Young, 1974; Hall and Bok, 1974), a process which may be of importance in visual pigment conversion in paired pigment species (see Chapter V, General Discussions).

In this study, vitamin A compositions of the pigment epithelia of goldfish and bullfrogs were measured and compared to the visual pigment compositions in the retinas (rainbow trout and leopard frogs did not possess sufficient vitamin A in their pigment epithelium for analysis). After 50 days of acclimation, rhodopsin-rich goldfish had predominately 3-dehydroretinol in their pigment epithelium. However, the rhodopsin-rich dorsal retinas of bullfrogs (also as a result of 40 days of light-temperature treatments) were associated with pigment epithelia having mostly vitamin A_1, although the percentages of vitamin A_2 in the pigment epithelium (25.9-42.4% vitamin A_2) remained higher than the porphyropsin percentages (3.8-7.3% porphyropsin) in the retinas. Since the vitamin A composition in the pigment epithelium does not always match the visual pigment composition in the retina, it is likely that the pigment epithelium does not regulate the visual pigment composition in the retina through the relative availability of its vitamin A_1 and A_2. Further experiments on the goldfish visual system showed that the percent 3-dehydroretinol values in the saponified pigment epithelium may match the porphyropsin ratio in the retina only after a prolonged period of acclimation (some 600 days, Chapter III). This suggests that the degree of resemblance between the vitamin A composition in the pigment epithelium and the visual

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bleaching and resynthesis of porphyropsin, follows a similar pathway.

pigment composition in the retina is dependent on the length of acclimation of the animal to a particular light and temperature regime.

Vitamin A available from other parts of the body, in addition to that stored in the pigment epithelium, may also affect visual pigment composition in an animal. For example, Beatty (1966) reported that in the adult coho salmon, rhodopsin and porphyropsin-rich retinas from fish caught at different locations were respectively associated with retinol-rich and 3-dehydroretinol-rich livers in the animals. In addition, Jacquest and Beatty (1972) induced significantly higher porphyropsin proportions in rainbow trout simply by feeding them with walleye (*Stizostedion vitreum*) liver extracts, a rich source of vitamin A_2. Furthermore, Beatty (1972) showed that the porphyropsin percentages in the retina and the 3-dehydroretinol percentages in the liver and in the pyloric caeca of juvenile kokanee were significantly increased after intraperitoneal injections of 3-dehydroretinol. These reports suggest that perhaps the vitamin A composition in the liver (which also stores vitamin A in addition to the pigment epithelium) should also be considered. In the present study, the vitamin A composition was analysed (as 3-dehydroretinol percentages in saponified livers) in all four species and compared to the visual pigment composition in the eye. The rainbow trout and the goldfish possessed mostly 3-dehydroretinol in their livers in proportions that were not correlated to the porphyropsin proportions in the retina. The two species of frogs (bullfrog and leopard frog), however, had almost exclusively retinol in their livers irrespective of the light and temperature treatments. These results suggest that the vitamin A composition in the liver may play a less

significant role (as compared to the vitamin A composition in the pigment epithelium) in the regulation of visual pigment composition in the retina.

CHAPTER II

VISUAL PIGMENTS AND VITAMINS A IN RAINBOW TROUT

Introduction

Rainbow trout *(Salmo gairdneri)* retinas contain a pair of scotopic visual pigments: a rhodopsin (λmax=502-503 nm) and a porphyropsin (λmax=527 nm) (Munz and Beatty, 1965). The relative proportions of these two visual pigments are known to vary according to the light and temperature environment to which the fish are subjected (Allen *et. al.*, 1973; Cristy, 1976; Tsin, 1976; McFarland and Allen, 1977).

Regardless of the numerous studies concerning the effects of the photic and thermal environments, little is known about the dynamics of changes in the visual pigment composition in paired pigment species. For example, when fish of different photopigment compositions are placed in the same light and temperature regime, will they eventually attain a similar and stable visual pigment composition? If so, will they reach such a 'steady-state' condition simultaneously? Allen (1971) attempted to answer the former by subjecting two groups of redside shiners (of different visual pigment compositions) to the same light environments. His results did not offer a comprehensive answer to the question because one group of shiners as pretreated with thyroxine and the effect of temperature was overlooked. Although visual pigment compositions of the two groups of shiners were similar on the 42nd day of acclimation, the establishment of a merged and stabilized steady-state visual pigment composition was not apparent (Allen, 1971, Fig. 18).

In this study, I have obtained experimental evidence showing how groups of rainbow trout, each with a different visual pigment composition, achieved steady-state when placed under specified light and temperature regimes. The rate of change in visual pigment composition was directly related to the magnitude of change so that different groups attained their steady-state simultaneously.

In subsequent experiments, some three hundred fish were used to establish the steady-state visual pigment compositions for 15 light and temperature regimes. The results (ranging from 13% to 92% porphyropsin) allow an accurate assessment (i.e. steady-state vs shifting visual pigment composition) as to how light and temperature influenced visual pigment composition in rainbow trout. The apparent predictability and reproducibility of these results also allow these data to serve as realistic criteria for the manipulation of rhodopsin/porphyropsin ratios in this species.

These animals do not have sufficient vitamins A in their pigment epithelium for measurements. The percent 3-dehydroretinol in their livers, however, did not bear any significant correlation to the porphyropsin proportions in the retinas.

Materials and Methods

Light and temperature control

The design of the light and temperature environment are similar to that described earlier (Tsin, 1976; Tsin and Beatty, 1977a). Two 20W fluorescent tubes (G.E. cool white, about 35 cm from water surface) were placed on the inside ceiling of an enclosed wooden lid housed on top of an aquamarine, fiberglass, rectangular tank (of an area of 75 X 55 cm^2, a depth of 35 cm with a water depth of 25 cm). The lid was painted white on the inside and the light/dark cycle was controlled by an automatic timer. The irradiance at water surface (see Appendix 8), as recorded from the remote probe of a spectroradiometer (ISCO, model SR) was 1.8 X 10^{15} photons/cm^2-sec (400-750 nm). The continuously dark tank had a similar lid painted black on the inside. The irradiance given by the red bulb (used only for daily inspection of the fish) was small (4.2 X 10^{13} photons/cm^2-sec, 400-750 nm).

Water temperature in the illuminated tank was controlled by a thermoregulator (Canlab C-series, T3445) in combination with a relay connected to solenoid valves on the warm (30^0C) and cold (5^0C) water lines. In the continuously dark tank, water temperature was controlled by a YSI Thermistemp temperature controller (Model 63SR, Yellow Springs Co., Ohio) similarly connected to solenoids on the warm and cold water lines. Tank temperatures were attained by the opening of either one of the two solenoid valves, alternately adding warm or cold water to the tank. In addition, air was constantly bubbled through the water to ensure aeration. Daily inspection showed that water temperature stayed constant within $\pm 0.5^0$C.

Analysis of visual pigments

Animals were always dark-adapted[1] for two hours before eye removal. Preparation and analysis of visual pigment extracts followed a standard procedure (Munz and Beatty, 1965; Beatty, 1969b; Tsin, 1976, Appendix 2; Knowles and Dartnall, 1977a). The retinas of both eyes of an individual were combined for extraction in 0.6 ml of 2% digitonin (Nutritional Biochemicals). Bleaching consisted of one 10 minute exposure to an orange light (610 nm). Using a template curve previously prepared (Munz and Beatty, 1965), the relative porphyropsin percentages (based on absorbance) were determined from the wavelength at the 50% point of the total difference spectrum (for details, see Tsin, 1976, Appendix 2). Conversion of the relative percentage of porphyropsin to molar porphyropsin percentages (based on molecular concentration) was made using values of 40600 and 30000 as molar extinction coefficients for rhodopsin and porphyropsin respectively (Hubbard, Brown and Bownds, 1971; Knowles and Dartnall, 1977b). Only molar percentage values are reported in this study.

Analysis of vitamins A in livers

The livers were freeze-dried, ground and saponified (under reflux) in 33 ml of 0.33M KOH in 94% ethanol for one hour at 70-80^{0}C. After chilling in ice, 40 ml of distilled water were added and the mixtures were shaken with two portions of 50 ml diethyl ether for vitamins A extraction. The diethyl ether fractions were then

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1 Holding an animal in darkness for two hours will facilitate the separation of the retina from the pigment epithelium in addition

separated and washed with two portions of 100 ml 0.5N KOH (to remove excess ether soluble acid soap, see p. 75, Freed, 1966) and 2 portions of 100 ml distilled water before being dried over anhydrous sodium sulfate.

After evaporation under oxygen-free dried nitrogen and reconstituted to 1 ml in chloroform, 10-100 µl were added to 1 ml of saturated antimony trichloride solution containing 50 µl of acetic anhydride for the Carr-Price procedures of vitamins A analysis (for details, see Hubbard *et. al.*, 1971). The vitamin A_2 (3-dehydroretinol) percentage values were calculated using two simultaneous equations (Hubbard *et. al.*, 1971).

Dynamics of visual pigment changes

Two hundred and forty rainbow trout (mean body weight, 33 g) were obtained form Allen's trout farm in Calgary (Alberta) in the fall, 1976. Upon arrival, they were divided into three groups of 80 each and subjected to the following light and temperature regimes for 45 days: 24L, 6^0C (group A); 12L/12D, 12^0C (group B) and 24D, 18^0C (group C). When the experiment began, half of the fish from each group were subjected to 24L, 6^0C and the other half to 24D, 18^0C. Five fish from each subgroup (i.e. a total of six subgroups) were sampled on days 0, 10, 20, 30, 40, 60 and 80. Groups held under 24D, 18^0C experienced some mortality resulting in early terminations of samplings. Fish were not fed during these and subsequent experiments. They were fed with commercial trout food in the holding period (EWOS pellets, Astra Chemicals Ltd., Ontario).

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to maximizing pigment yield due to visual pigment regeneration in the dark.

Steady-state visual pigment compositions

Two hundred and fifty rainbow trout (mean body weight, 28 g) were obtained from Sam Livingston Trout Hatchery in Calgaty (Alberta) in the summer of 1977. After a month in an indoor holding tank (10^0C, room light), they (mean=40% porphyropsin, n=22) were divided into 11 groups and subjected to different light and temperature regimes for a period of 60 days. These conditions were: 24L, 12^0C; 24L, 18^0C; 12L/12D, 6^0C; 12L/12D, 12^0C; 12L/12D, 18^0C; 6L/18D, 6^0C; 6L/18D, 12^0C; 6L/18D, 18^0C; 24D, 6^0C; 24D, 12^0C and 24D, 18^0C. Unfortunately, the failure of a relay on the 47^{th} day of the experiment resulted in a decrease in the temperature on one tank (6L/18D, 12^0C). The results of this group are consequently not reported.

In the winter of 1978, some fifty rainbow trout of similar body size (mean body weight, 45 gm) and initial visual pigment composition (45.5% porphyropsin, n=12) were used to repeat the 6L/18D, 12^0C group with three additional groups added to complete this study (18L/6D, 6^0C; 18L/6D, 12^0C and 18L/6D, 18^0C). These trout were obtained from Four Springs Trout Ranch at Bluffton (Alberta).

Statistical analysis

Employing either the Student's T test or the analysis of variance, differences at the 5% level are recognized as significant.

Results

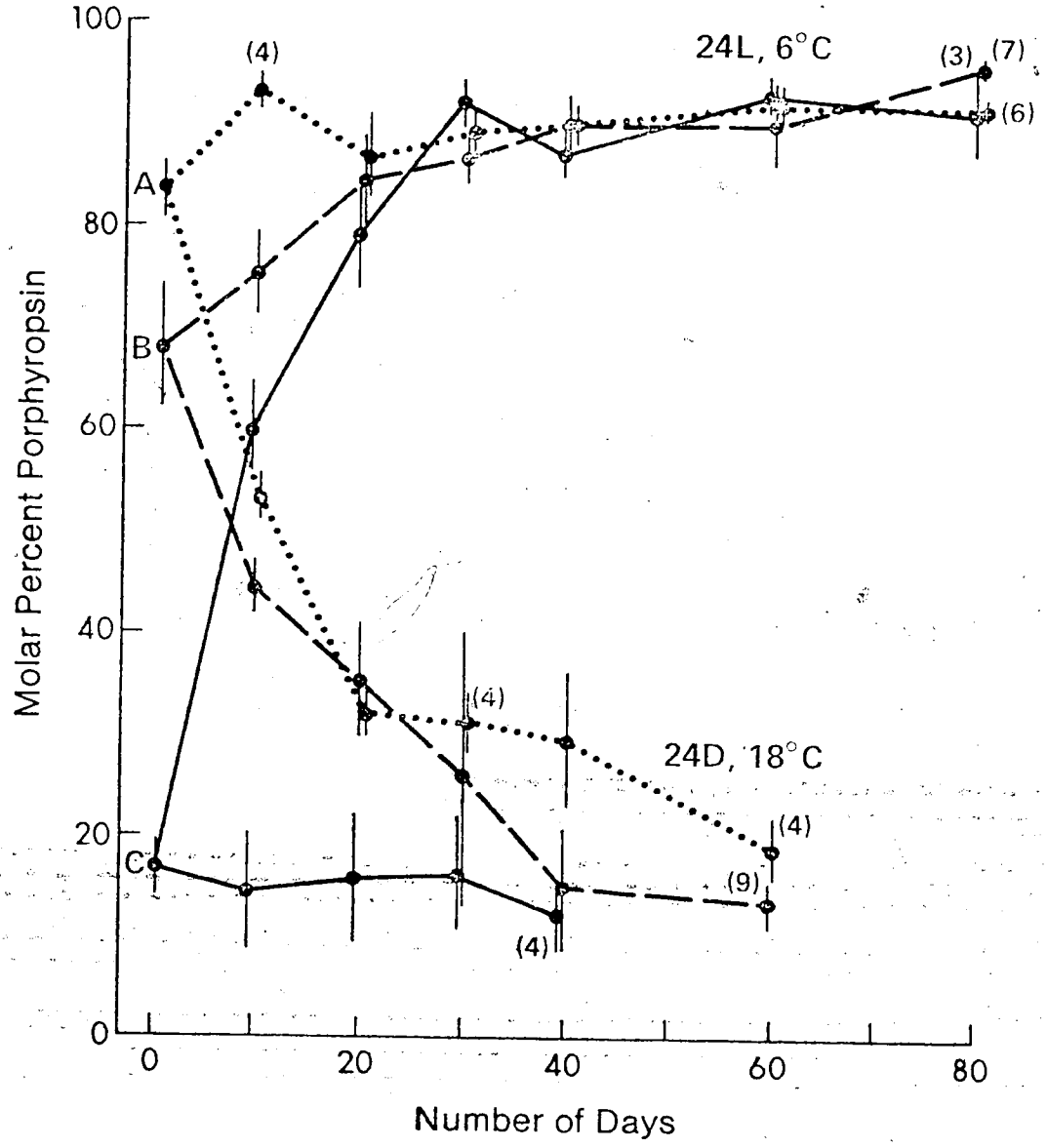
Dynamics of visual pigment changes

After 45 days of pretreatment, groups A (24L, 6^{0}C), B (12L/12D, 12^{0}C) and C (24D, 18^{0}C) had different initial porphyropsin proportions (A, 83.4%; B, 67.8%; C, 16.5%, Fig. 1, see also Appendix 1). When these fish were placed in 24L at 6^0, visual pigment ratios of groups B and C fish gradually merged into a stablized steady-state. Group C fish shifted their visual pigment composition towards predominately porphyropsin at a much faster rate than those of group B. This resulted in the almost simultaneous attainment of steady-state in both groups within 30-40 days in their new environment. The change in porphyropsin proportions was not constant with time. In fact, it decreased as the visual pigment composition approached steady-state. This is clearly demonstrated in group C fish where the increases were 43 molar percent from day 0 to 10, 20 molar percent from day 10 to 20 and 13 molar percent from day 20 to 30.

When fish were held under 24D at 18^{0}C, the visual pigment compositions of groups A and B moved towards a steady-state in a similar fashion as described earlier for groups B and C held in 24L, 6^{0}C. Unfortunately, mortality associated with holding fish in constant darkness (24D) resulted in earlier termination of samplings (Fig. 1). The leveling off of porphyropsin proportions of group A fish after the 20th day of experiment may be due to a failure of a relay resulting in a 24 hour temperature decrease. The results from group B fish, nevertheless, indicated that a steady-state was attained at a period of 40-50 days of acclimation (Fig. 1).

Fig. 1. Dynamics of visual pigment change in rainbow trout

Pretreatment consisted of conditioning three groups of fish (80 each) to three light and temperature regimes for 45 days (24L, 6^0C, group A; 12L/12D, 12^0C, group B and 24D, 18^0C, group C). When the experiment began, half of each of groups A, B and C were subjected to 24L, 6^0C and the other half, to 24D, 18^0C. Regular sampling involved five fish per group unless otherwise indicated in the figure. Dotted, broken and solid lines respectively join results from groups A, B and C. Means± 1 S.E. are indicated.


100
80
60
40
20
0
0
20
40
60
80
Molar Percent Porphyropsin
Number of Days
24L, 6°C
24D, 18°C
A
B
C
(4)
(3)
(7)
(6)
(4)
(4)
(9)
(4)

Since steady-state is established within 30-50 days of acclimation and both groups A (in 24L, 6^0C) and C (in 24D, 18^0C) had been in their respective conditions for 45 days before the experiment began, their visual pigment compositions were likely at steady-state at the start of the experiment. Their porphyropsin proportions during the experiment, hence allow an assessment of the 'stability' of these steady-states. Both groups A (in 24L, 6^0C) and C (in 24D, 18^0C) clearly demonstrated that their visual pigment compositions remained virtually unchanged throughout their respective 80 and 40 days of the experiment (Fig.1).

Steady-state visual pigment compositions

From the previous experiment, it is concluded that the change of visual pigment composition in rainbow trout is not constant with time. This implies that a shifting, instead of a steady-state, visual pigment composition may be a less reliable index to estimate the effect of light and temperature. Consequently, porphyropsin ratios of trout held under 15 different regimes for 60 days (i.e., assuming steady-state is reached and maintained within 60 days of acclimation) were determined in an attempt to elucidate the light and temperature effects on the visual pigment composition in rainbow trout.

The manipulation of light and temperature resulted in a wide range of porphyropsin percentages (Fig. 2a and b, 13% in 24D, 18^0C to 92% in 24L, 6^0C, see also Appendix 2). Lower water temperatures (6^0C, 12^0C) in comparison to higher temperatures (12^0C, 18^0C) were consistently associated with higher porphyropsin proportions at each of the light conditions (i.e., 24L, 18L, 12L, 6L and 24D, Fig. 2a).

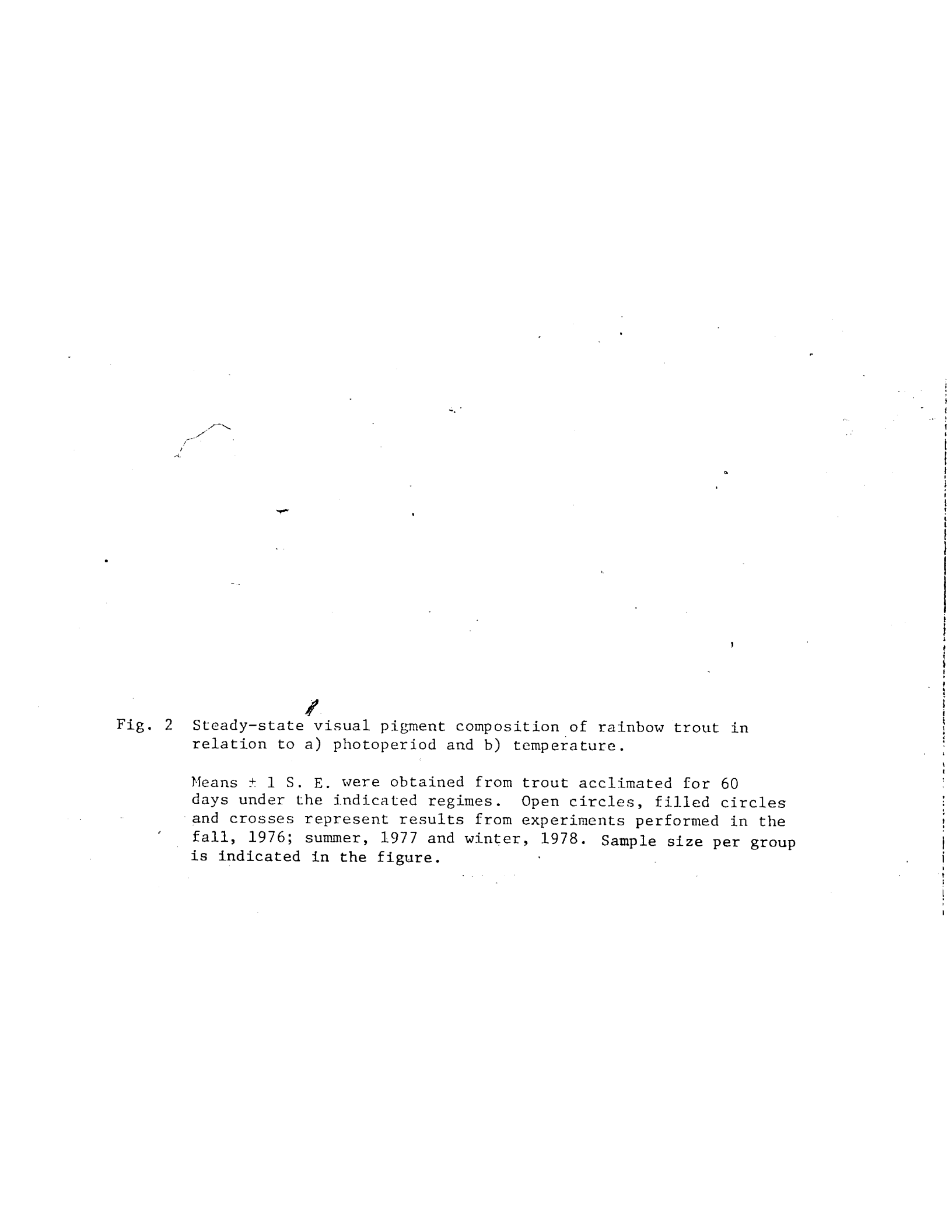
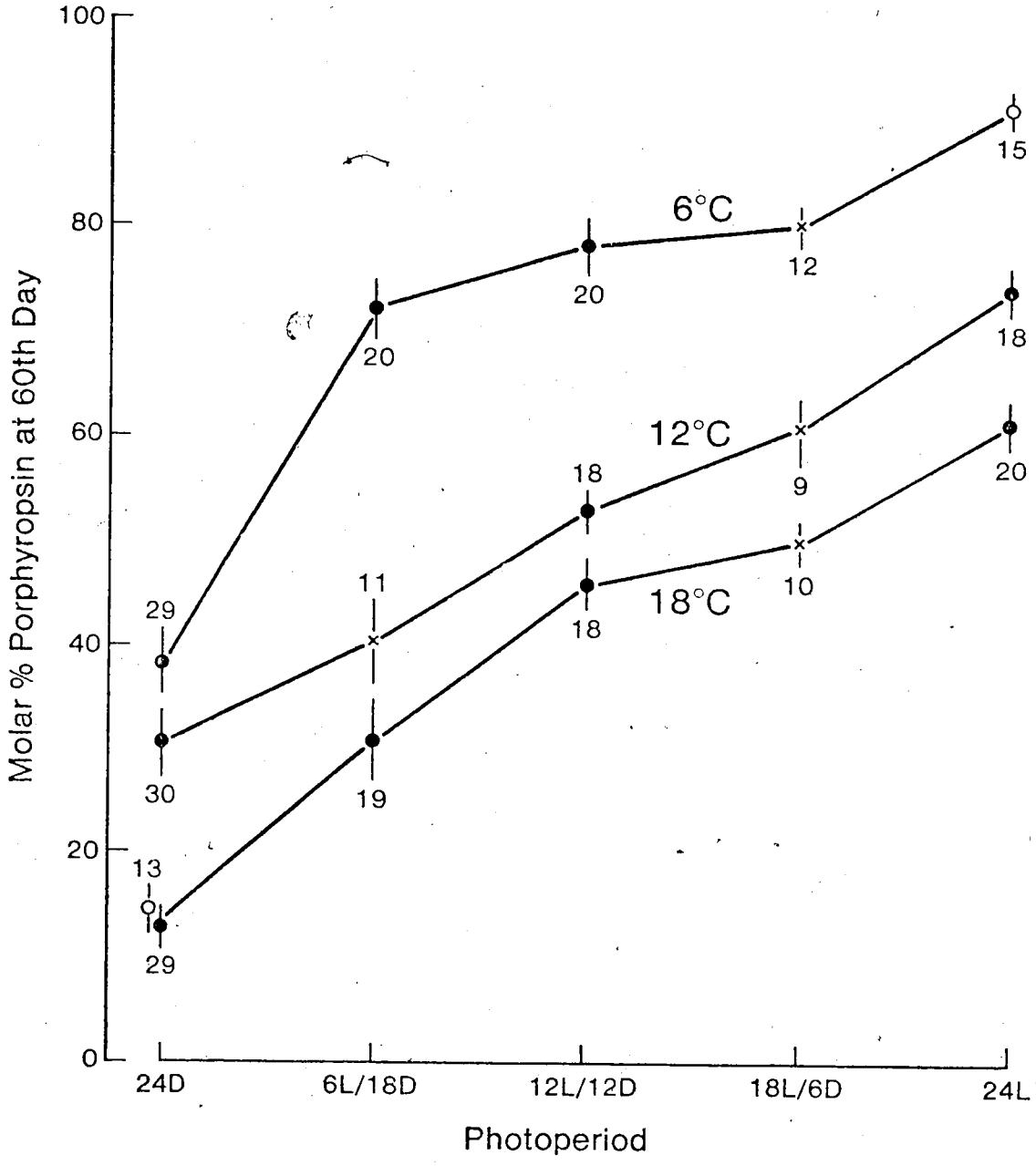
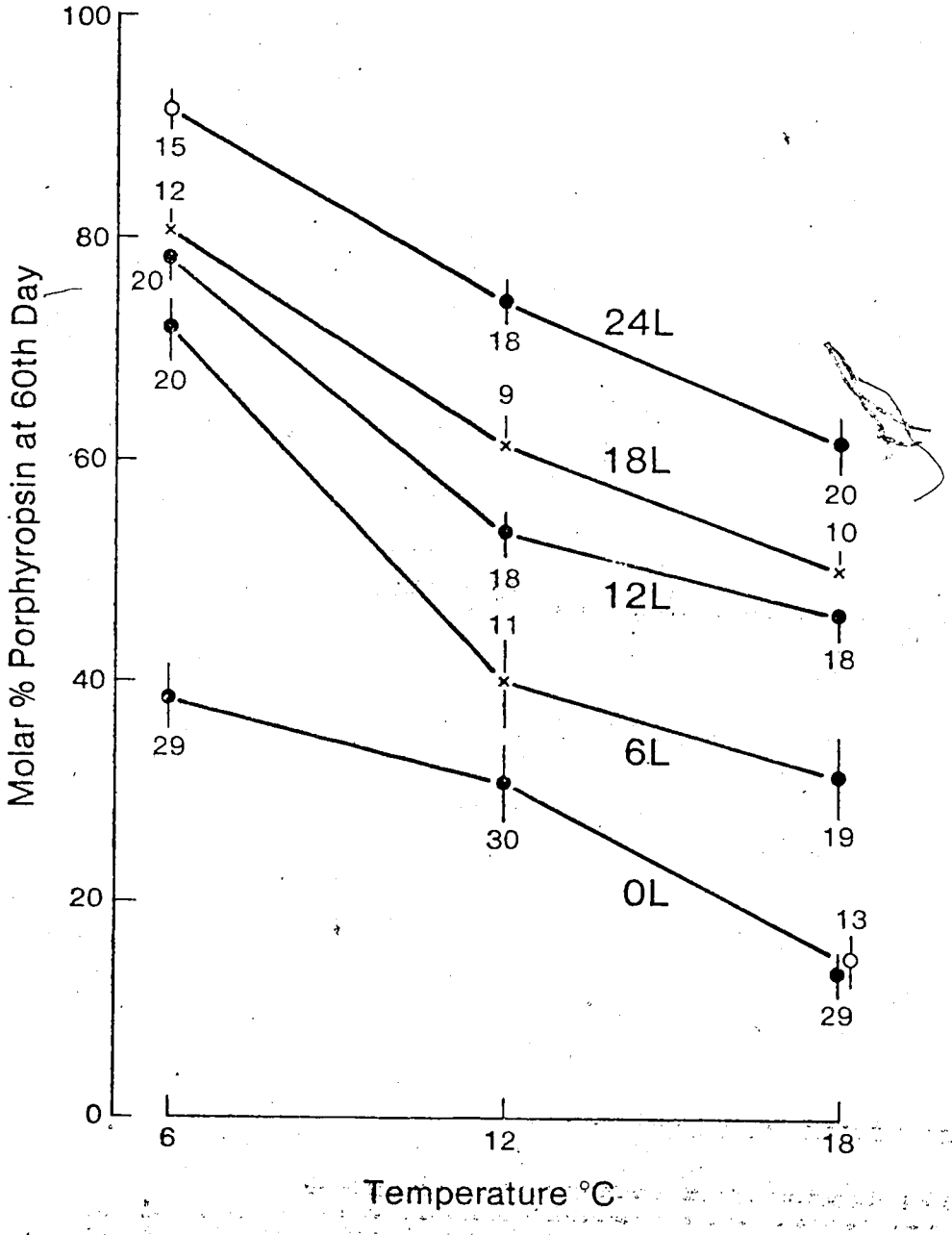


Fig. 2 Steady-state visual pigment composition of rainbow trout in relation to a) photoperiod and b) temperature.

Means ± 1 S. E. were obtained from trout acclimated for 60 days under the indicated regimes. Open circles, filled circles and crosses represent results from experiments performed in the fall, 1976; summer, 1977 and winter, 1978. Sample size per group is indicated in the figure.


100
80
60
40
20
0
Molar % Porphyropsin at 60th Day
6°C
12°C
18°C
15
20
12
20
18
9
20
18
10
11
18
29
30
19
13
29
24D
6L/18D
12L/12D
18L/6D
24L
Photoperiod


100
80
60
40
20
0
Molar % Porphyropsin at 60th Day
15
12
20
20
18
9
18
11
29
30
20
10
18
29
24L
18L
12L
6L
0L
13
6
12
18
Temperature °C

At any one of these temperatures (i.e. 6^0C, 12^0C and 18^0C), an increase in the length of the light period always resulted in an increase in porphyropsin ratios (Fig. 2b). In some instances, the porphyropsin proportions appeared to be directly related to the length of light period (from 24D to 24L at 12^0C and 18^0C, Fig. 2a) and to temperature (at 24L and 18L, from 6^0C to 18^0C, Fig. 2b). However, this relationship was not observed at a lower temperature (at 6^0C, from 24D to 24L, Fig. 2a) and for shorter photoperiods (at 24L, 6L and 0L, from 6^0C to 18^0C, Fig. 2b). The agreement of results from the experiments carried out in three different years clearly demonstrated the predictability (results from all groups studied in the winter, 1978 appear to agree with results from groups studied in 1976 and 1977) and reproducibility (results of 24D, 18^0C from the fall, 1976 and the summer, 1977 are similar, Fig. 2a) of the steady-state visual pigment composition in rainbow trout.

Vitamin A composition in the livers

The environmental factors did not exert any significant influence on the liver vitamin A composition in trout (see Appendix 1) which had significant difference in visual pigment composition in response to light and temperature (Fig. 1). Liver samples from trout (Appendix 2) were stored for a prolonged period of time (up to several months) and thus experienced some destruction of vitamin A. The results from successful determinations, however, indicated that there was no significant difference of 3-dehydroretinol percentages in livers amongst treatment groups (see Appendix 2).

Discussion

The visual pigment composition in rainbow trout is determined mainly by the light and temperature environment. When the fish are transferred to a new environment, the visual pigment composition of the fish will shift to that determined by the new light and temperature regime. This change is likely completed within the first 30-50 days, irrespective of the magnitude of the change. This is because the rate at which visual pigment composition changes varies proportionately with the amount of change. The fact that the change of porphyropsin proportions is not constant with time suggests that a stabilized steady-state visual pigment composition may be appropriate in the study of light and temperature effects. The steady-state visual pigment compositions show that light and temperature effects are interdependent (using two-way analysis of variance, a significant interaction was found between the two effects) and it is not likely that any simple equation, employing photoperiod and temperature, can accurately predict porphyropsin ratios in rainbow trout.

Cristy (1976) has formulated an equation to predict visual pigment compositions in rainbow trout held for 4 weeks in a given light intensity and temperature. The equation entails the use of a fixed photoperiod (11.5L/12.5D) and a specific blue light source. The assumptions on which this equation was based (that the change of visual pigment composition vs. the logarithmic change of light intensity, measured at 12^0C, is independent of temperature and that the change of visual pigment composition vs. the change of temperature, at a light intensity of 1.773×10^{13} photons/cm^2-sec, is independent of

light intensity), however, were not extensively tested. The results of the present study point out, at least, that the change of the steady-state visual pigment composition vs. the change of temperature appear to be dependent on the photoperiod (Fig. 2b) and that the change of the steady-state visual pigment composition vs. the change of photoperiod appear to be dependent on temperature (Fig. 2a). In view of the apparent predictability of results in the present study (see Results) and the readily reproducible light and temperature environments (see Methods), the data presented in Fig. 2a and b may suggest an alternative for Cristy's equation in the manipulation of porphyropsin proportions in rainbow trout.

In an earlier study, McFarland and Allen (1977) reported effects of light and temperature on the visual pigment composition of the common shiner and the rainbow trout. They subjected these animals to three photoperiods (24L, 12L/12D and 24D) at two different temperatures (4.5^{0}C and 14^{0}C) for 2 and 4 weeks. After 4 weeks of acclimation, groups of trout (and shiners) held at the lower temperature (4.5^{0}C) did not have significant differences in their porphyropsin percentages, irrespective of the different light treatments (Fig. 1a and b, McFarland and Allen, 1977). Trout held in a higher temperature (14^{0}C) had lower porphyropsin levels in comparison to those held at 4.5^{0}C, in agreement with results of the present study. At 14^{0}C, rainbow trout held under 12L/12D and 24D appeared to have comparable porphyropsin levels which were lower than those held under 24L (Fig. 1a, McFarland and Allen, 1977). On the other hand, common shiners held at 14^{0}C under 12L/12D had porphyropsin levels significantly lower than those held at 24L and

24D (Fig. 1b, McFarland and Allen, 1977). Based on these results, they concluded that 'in cyclic light, both species achieved their highest and their lowest porphyropsin levels (Fig. 1).' This conclusion appears inappropriate for rainbow trout based not only on their own results (Fig. 1a, McFarland and Allen, 1977) but also on the results of the present study (Fig. 2), although the present study employed a different light source (fluorescent tubes) than the tungsten light used in their experiments (see Appendix 8 for the difference in light quality of the two light sources). This difference in light quality, in addition to a difference in light intensity between the present study (1.8×10^{15} photons/cm^2-sec, 400-750 nm) and their study (1.6×10^{14} photons/cm^2-sec, 400-700 nm) may be responsible[1] for the relatively low levels of porphyropsin in their rainbow trout (less than 50% porphyropsin) held at a low temperature (4.5^0C) and under a long photoperiod (24L) (see Fig. 2a and b).

In addition to the light and temperature factors, several hormones (thyroxine, T4; tri-iodothyronine, T3, bovine TSH and ovine prolactin) have also been shown to be effective in changing the proportions of rhodopsin and porphyropsin in fishes (Munz and Swanson, 1965; Beatty, 1969b, 1972; Allen, 1971, 1977; Jacquest and Beatty, 1972; Cristy, 1974; Tsin and Beatty, 1977b; McFarland and Allen, 1977). Consequently, an environmental parameter (such as

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[1] Cristy (1976) reported that an increase in the light intensity resulted in an increase in porphyropsin levels in rainbow trout.

temperature) may have affected visual pigment composition via the hypothalamus-pituitary-thyroid axis (for the possible relationship of temperature and thyroid activity in fishes, see reviews of Gorbman, 1969; Etkin and Gona, 1974). In earlier studies, both Tsin and Beatty (1977b) and Allen (1977), using tetralute kits (Ames Co.), measured T4 levels in the blood of T4 treated rainbow trout and rainbow trout held under different light and temperature regimes. Although the exogenous T4 induced significantly higher levels of both circulatory T4 and proportions of porphyropsin, the blood T4 levels did not correlate to the levels of porphyropsin in fish held under different light and temperature environments (Tsin and Beatty, 1977b; Allen, 1977).

Whether or not light and temperature effects are mediated through hormonal mechanism, their effects on the visual pigment composition in trout are apparent. Paired visual pigment fishes have been reported to exhibit changes in visual pigment composition in their natural habitats (for review, see Beatty, 1975a). The present study reveals two fundamental factors which may induce visual pigment conversion taking place in changing light and temperature environments. Further studies on the influence of light intensity, light quality and other factors (such as salinity and sexual maturation) may eventually lead to the understanding of the physiological and ecological significance of visual pigment changes in paired visual pigment species.

The ability to manipulate visual pigment composition employing appropriate light and temperature regimes provides an opportunity to investigate the mechanism involved in visual pigment conversion in paired visual pigment fishes. This relates especially to the role

played by the pigment epithelium which lies adjacent to the photoreceptor outer segments where visual pigment changes occur. Unfortunately, rainbow trout do not possess sufficient vitamin A in their pigment epithelium for analysis. The fact that vitamin A compositions in the livers were not affected by light and temperature influence suggests a more complex role of the liver in the storage and supply of prosthetic groups for pigment synthesis in the retinas.

CHAPTER III

VISUAL PIGMENTS AND VITAMINS A IN GOLDFISH

Introduction

In an extensive survey on visual pigments of fresh water fishes, Schwanzara (1967) reported that goldfish, *Carassius auratus*, possess a single scotopic visual pigment: a porphyropsin with an absorbance maximum at 522 nm. Liebman (1972), using microspectrophotometry, has also confirmed the presence of a visual pigment with λmax at 522 nm in goldfish rods. Subsequently, Bridges (1973, 1975) concluded that goldfish retinas contain only porphyropsin and the pigment epithelium, only vitamin A_2. In addition, several investigators (Liebman and Entine, 1964; Marks, 1965; Harosi and MacNichol, 1974; Stell and Harosi, 1976) have shown that goldfish have three separate cone pigments and Harosi and MacNichol (1974) have indicated that the prosthetic group for these pigments is 3-dehydroretinal. Consequently, it has generally been accepted that goldfish have rod and cone pigments based solely on 3-dehydroretinal.

The earlier study on rainbow trout (Chapter II) shows that the visual pigment composition in a paired pigment animal can be predictably manipulated using light and temperature. Assuming that goldfish is also a paired visual pigment fish[1], the rhodopsin/porphyropsin ratio in this animal may also respond to environmental treatments. Since higher water temperature always favors higher

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[1] See Rhodopsin in Grassyfork Goldfish in Methods for the basis of this assumption.

rhodopsin proportions in paired pigment fishes, irrespective of the animal's response to light by an increase or a decrease of porphyropsin proportions (Allen and McFarland, 1973; Cristy, 1974; Tsin, 1976; Tsin and Beatty, 1977a, McFarland and Allen, 1977), it would be of interest to see if a detectable proportion of rhodopsin can be 'induced' by subjecting goldfish to a high water temperature (28^0C) at an arbitrarily chosen photoperiod (16L/8D) and light intensity (see Methods, for details). This assumption was fully confirmed in an experiment in the goldfish visual system (Tsin and Beatty, 1978, see also Methods) showing that goldfish is a paired pigment species possessing a porphyropsin (λmax=522 nm) and a rhodopsin (λmax=499 nm).

Additional experiments on the goldfish visual system showed that the relative proportion of rhodopsin and porphyropsin in the retina of goldfish can be altered by photoperiod, light intensity, temperature and exogenous thyroxine. By employing an optimal photoperiod, an optimal light intensity[1] and a high water temperature, the visual pigment composition in the goldfish retina may change from predominately porphyropsin to predominately rhodopsin in 50 days. The pigment epithelia of these goldfish were always high in 3-dehydroretinol proportions irrespective of the visual pigment composition in the retina. Serial sampling of goldfish over a long period indicated that although the goldfish retina may accomplish an almost complete turnover from porphyropsin to rhodopsin

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[1] Tungsten light bulbs were used in this study because the light intensity delivered by this light source can be conveniently manipulated using different size light bulbs (i.e. 7.5W, 15W, 60W etc.) or by dimming with a rheostat. The difference between the quality of light from a tungsten and a fluorescent source is explained in Appendix 8.

in about 50 days, replacement of 3-dehydroretinol by retinol in the pigment epithelium may require up to 600 days. The relative availability of the two stored vitamins A in the pigment epithelium, therefore, does not determine the visual pigment composition in the retinas as suggested by previous investigators (Reuter *et. al.*, 1971; Bridges and Yoshikami, 1970b).

Materials and Methods

Light and temperature control

The controlled light and temperature regimes were similar to those described earlier (Chapter II). All wooden lids housed on top of the rectangular fiberglass tanks (120 liter capacity, see Chapter II) were painted black on the inside. The photoperiod was controlled by an automatic timer. The light intensity was manipulated by using different size incandescent light bulbs (i.e. 7.5W, 15W and 60W) located at the ceiling of the lid (the light bulb was approximately 30-35 cm from water surface). Light intensity was measured from 400 nm-750 nm using the remote probe of a spectroradiometer (ISCO). The water temperature was controlled by a thermoregulator in combination with a relay connected to solenoids on the warm or cold temperature inlets as previously described (Chapter II). Dechlorinated water constantly flowed into the tank and air was constantly bubbled through the water. The temperature of the static water (for thyroxine treatment, see later paragraphs) was controlled by running temperature controlled water through an enclosed stainless steel coil immersed into the tank. Animals were fed daily with commercial fish food (EWOS pellets, Astra Chemicals Ltd., Ontario).

Analysis of visual pigments and vitamin A

The analysis of visual pigments followed a standard procedure (see Chapter II). The eyes of an individual were always removed at the end of the dark phase of the light-dark cycle (for the constantly illuminated group, after a dark adaptation period of two hours).

The two retinas of an individual were combined to prepare the 0.5 ml digitonin extract. Each extract was totally bleached with ten minutes exposure to an orange light (610 nm), except as otherwise indicated. The relative porphyropsin percentage derived from the total difference spectrum was converted to the molar porphyropsin percentage using values of 40600 and 30000 as molar extinction coefficients for rhodopsin and porphyropsin respectively (Hubbard *et. al.*, 1971).

The vitamin A in the pigment epithelium was extracted and saponified following the procedures of Bridges (1975). In essence, the tissues were freeze-dried overnight and extracted with petroleum ether (B.P. 37.4-58.8^{0}C) (2 portions of 3 ml each), which was then dried under oxygen-free dried nitrogen. The residue was saponified for 20 min. at 55^{0}C in 4 ml of 0.33M KOH in 94% ethanol. After chilling in ice, a small amount of distilled water was added and the mixture was extracted with petroleum ether (2 portions of 10 ml each). The petroleum ether extract was then washed with distilled water (3 portions of 10 ml each) and dried over anhydrous sodium sulfate.

After evaporation under nitrogen and reconstitution to 1 ml in chloroform, 10 µl of the sample were added to 1 ml saturated $SbCl_3$ containing 50 µl of acetic anhydride for the colorimetric analysis of the two vitamins A (for the Carr-Price procedures, see Hubbard *et. al.*, 1971). The relative proportions of the two vitamins were calculated using simultaneous equations (Hubbard *et. al.*, 1971).

The liver vitamin A was saponified, extracted and analysed in a similar way as described previously (Chapter II).

Rhodopsin in Grassyfork Goldfish

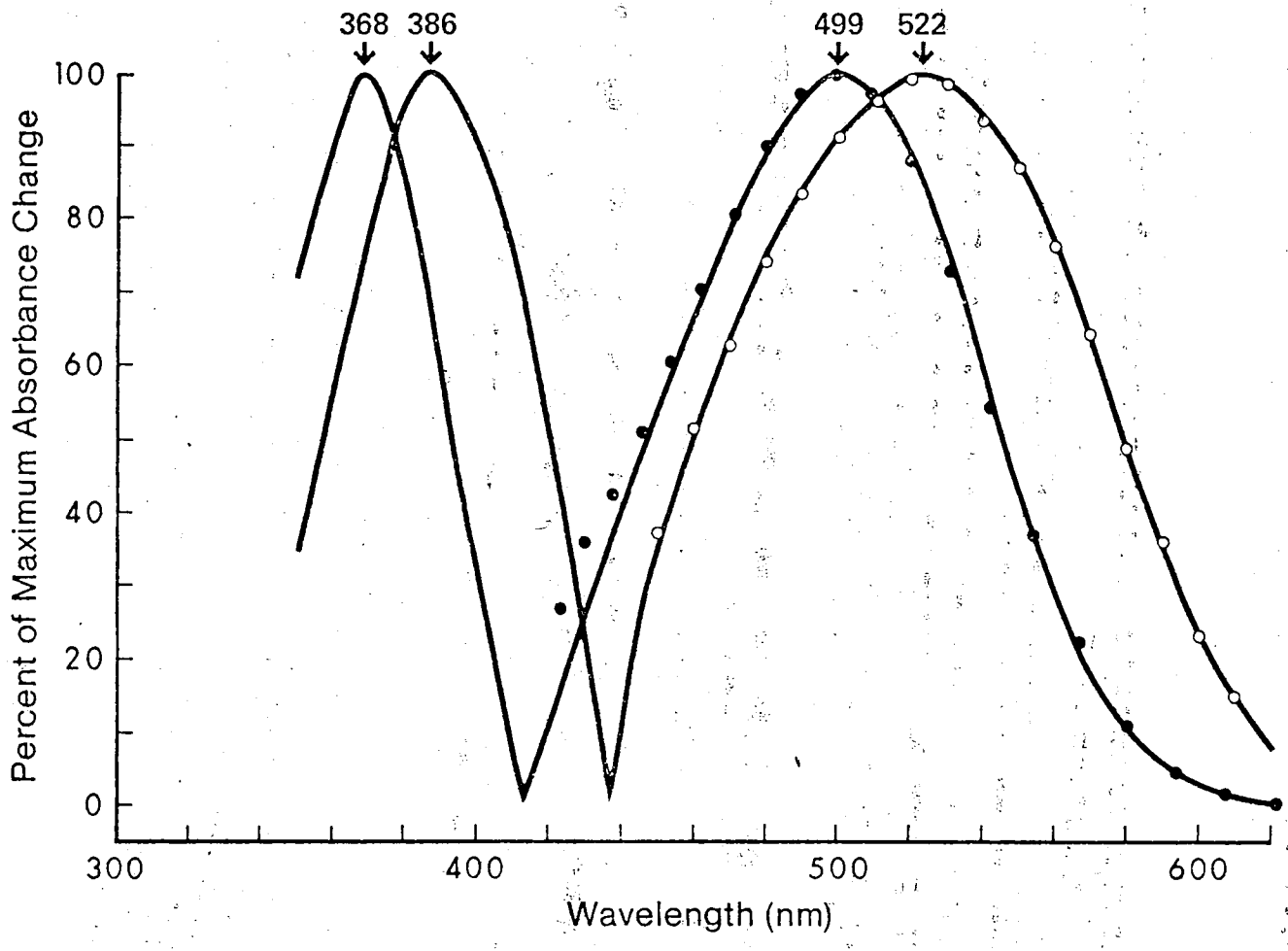
Two year old comet goldfish (24-45 gm) were obtained from Grassyfork Fisheries (Martinsville, Indiana 46151, U.S.A.) in November, 1977. According to the supplier, these fish were from a homogenous group derived from an inline breeding program. They were hatched, grown and maintained in outdoor ponds (under natural photoperiod) with water temperatures ranging from 30^0C in the summer to less than 4^0C in the winter (under ice cover). On arrival, the fish were in 12^0C water.

Visual pigment extracts from ten goldfish sampled upon arrival contained a porphyropsin of λmax=522 nm (Fig. 3), in agreement with previous studies (Schwanzara, 1967; Liebman, 1972). There was, however an indication of a small amount of another visual pigment with maximun absorbance near 500 nm, suggestive of a rhodopsin. In the earlier study on rainbow trout, an increase of rhodopsin proportion was induced by warmer temperatures (Chapter II). Consequently, a group of twelve goldfish were subjected to a temperature of 28^0C and a photoperiod of 16L/8D (under a 60W tungsten bulb, 70 cm above the water surface) in a 250 l, aquamarine, fiberglass holding tank. Fish were sampled on the 30^{th} and 36^{th} days.

Partial bleaching experiments on the visual pigment extracts from these fish revealed that the proportion of the second pigment had increased relative to that of the porphyropsin. The difference spectra from the final bleach with 610 nm light, when re-scaled to a percentage of each difference spectrum's maximum absorbance change, were a set of congruent curves with λmax at 499 nm and an oxime photoproduct with maximum absorbance at 368 nm, indicating that

Fig. 3 Difference spectra, measured in the presence of hydroxylamine, of rhodopsin (λmax=499 nm) and porphyropsin (λmax=522 nm) in the goldfish.

The bleaching protocol consisted of an initial one minute exposure to 675 nm light, a 4 minute exposure to 660 nm light and a final 10 minute exposure to 610 nm light. The curve for pigment 522 is the mean result from the initial bleaching (675 nm light) of three extracts from fish having more than 90% porphyropsin. The photoproduct with λmax at 386 nm is indicative of 3-dehydroretinal oxime, thus pigment 522 is a porphyropsin. The curve for pigment 499 is the mean result from the final bleaching (610 nm light) of three extracts from fish having less than 40% porphyropsin. The photoproduct with λmax at 368 nm is indicative of retinal oxime, thus pigment 499 is a rhodopsin. The open and filled circles, respectively represent the theoretical difference spectrum for a porphyropsin with λmax at 522 nm (nomogram of Munz and Schwanzara, 1967, for porphyropsins) and the theoretical absorbance spectrum for a rhodopsin with λmax at 499 nm (revised nomogram of Dartnall for rhodopsin, Wyszecki and Stiles, 1967).


368
386
499
522
100
80
60
40
20
0
Percent of Maximum Absorbance Change
300
400
500
600
Wavelength (nm)

retinal is the prosthetic group (Hubbard *et. al.*, 1971) and that the second visual pigment is a rhodopsin (Fig. 3). This is clear evidence that Grassyfork goldfish possess a rhodopsin with an absorbance maximum at 499 nm.

To further substantiate the λmax of the goldfish rhodopsin, the equation of Dartnall and Lythgoe (1965) predicts that λmax for a rhodopsin, having the same opsin as a porphyropsin with λmax at 522 nm, is at 499 nm. Carr-Price tests (Hubbard *et. al.*, 1971) on saponified pigment epithelium and bleached retinas from the goldfish held at 28^{0}C (16L/8D) provided distinct peaks at 616 nm and 690 nm, indicating the presence of both retinol and 3-dehydroretinol in the eye tissue. The pure difference spectra of the two pigments (Fig. 3) were used to construct a series of intermediate curves representing various mixtures (Munz and Beatty, 1965). Using these curves, the relative proportion of the two pigments was estimated for individual fish. All fish sampled on arrival had in excess of 85% porphyropsin (mean, 90.8%, n=10) whereas all fish held at 28^{0}C for 30-36 days had less than 70% porphyropsin (mean, 48.7%, n=12) with six having less than 50%.

Effect of photoperiod, light intensity and temperature

Goldfish were obtained from Grassyfork Fisheries in April, 1978. Upon arrival, ten fish (mean body weight, 45 gm) were analysed for visual pigment composition in the retina (93.3% porphyropsin) and vitamin A composition in the pigment epithelium (88.7% 3-dehydroretinol). The remainder was then divided into nine groups of 13 fish each and subjected to the following photoperiod, light intensity, and temperature condition for 50 days: 24L, 7.5W, 30^{0}C;

16L/8D, 7.5W, 30°C; 8L/16D, 7.5W, 30°C; 24D, 30°C; 16L/8D, dimmed 7.5W, 30°C; 16L/8D, two 15W, 30°C; 16L/8D, two 60W, 30°C; 16L/8D, 7.5W, 20°C; 16L/8D, 7.5W, 10°C. The light intensities of these bulbs were (as measured at water surface with a spectroradiometer scanning from 400-750 nm): 7.5W, 6.0×10^{13} photons/cm^2-sec; two 15W, 5.0×10^{14} photons/cm^2-sec; two 60W bulbs, 2.4×10^{15} photons/cm^2-sec and the dimmed 7.5W bulb, 5.9×10^{12} photons/cm^2-sec (see Appendix 8).

Effect of exogenous thyroxine

Goldfish were obtained from a second supplier (Hartz Mountain Pet Supplies) in Calgary (Alberta) in April, 1978. The supplier certified that they had recently obtained these fish from Ozark Fisheries in Stoutland, Missouri 65567. Partial bleaching experiments confirmed that these goldfish possess a porphyropsin with a λmax at 522 nm and a rhodopsin with a λmax at 499 nm, in agreement with the values for goldfish from Grassyfork Fisheries.

Upon arrival, ten goldfish were analysed for visual pigment composition in their retinas (86.6% porphyropsin) and vitamin A composition in their pigment epithelium (91.8% 3-dehydroretinol). The remainder were placed in two holding tanks (100 liter capacity, fiberglass tanks without wooden lids) having a 7.5W bulb projecting 40 cm overhead and a photoperiod of 16L/8D at 28°C. After 29 days of acclimation, four fish from each tank were analysed for visual pigment composition by partial bleaching experiments (38.0% porphyropsin, n=8). No significant difference in visual pigment composition was found between fish from the two holding tanks. The percentages of 3-dehydroretinol in the pigment epithelium of these fish, unfortunately, were not

measured due to a failure of the freeze-dryer.

Thyroxine treatment commenced on the 30th day of acclimation. L-thyroxine (Sigma Chemicals), dissolved in a small amount of 0.1M NaOH, was introduced to the static tank water at a concentration of 100 µg L-thyroxine per liter. Half of the tank water of both control and treatment tanks was drained and replenished with dechlorinated water (28^{0}C) every other day with sufficient L-thyroxine added to the treatment tank to provide a concentration of 100 µg per liter. Goldfish were sampled on the 10th and 20th days of treatment.

Effect of long term acclimation

Goldfish from Grassyfork Fisheries were placed in a holding tank (without a wooden lid on top) with a 15W light bulb projecting overhead at a photoperiod of 16L/8D and water temperature of 28^{0}C. These goldfish were sampled at days 0, 50, 150, and 300.

Results

Effect of photoperiod, light intensity and temperature on visual pigment composition (Grassyfork Goldfish)

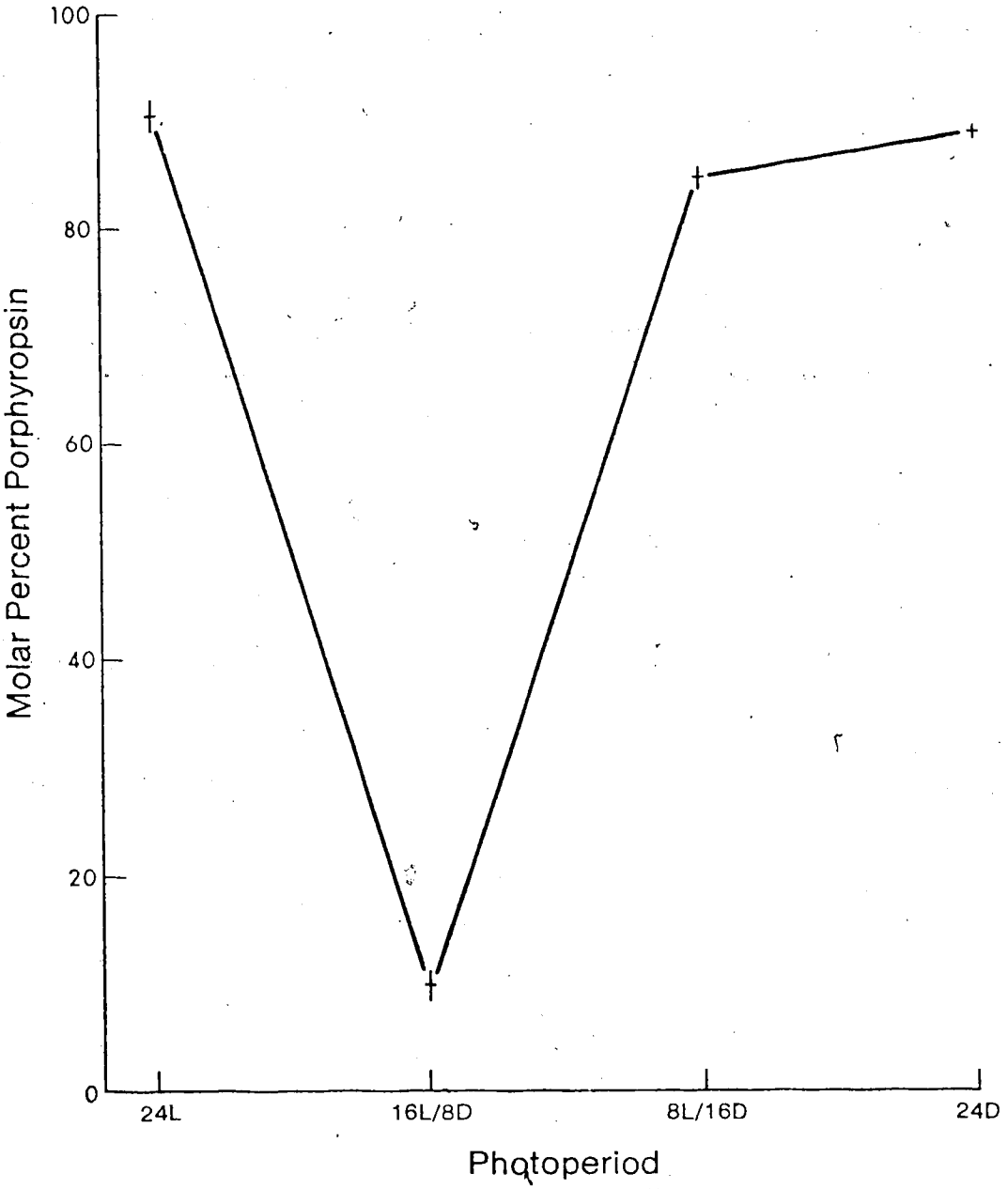
The effects of photoperiod, light intensity and temperature on the visual pigment composition are summarized in Fig. 4a, b and c (Appendix 3). At 30^0C and at a light intensity of 6.0×10^{13} photons/cm^2-sec, 400-750 nm (i.e. by a 7.5W light bulb), those fish in 24L, 8L/16D and 24D had high porphyropsin proportions (90.5%, 84.6% and 88.5% respectively, whereas those in 16L/8D had high rhodopsin proportions (90.2%, Fig. 4a). The 16L/8D photoperiod (at the specified temperature and light intensity) therefore appears to be an optimum condition in the conversion of visual pigments from predominately porphyropsin to mostly rhodopsin.

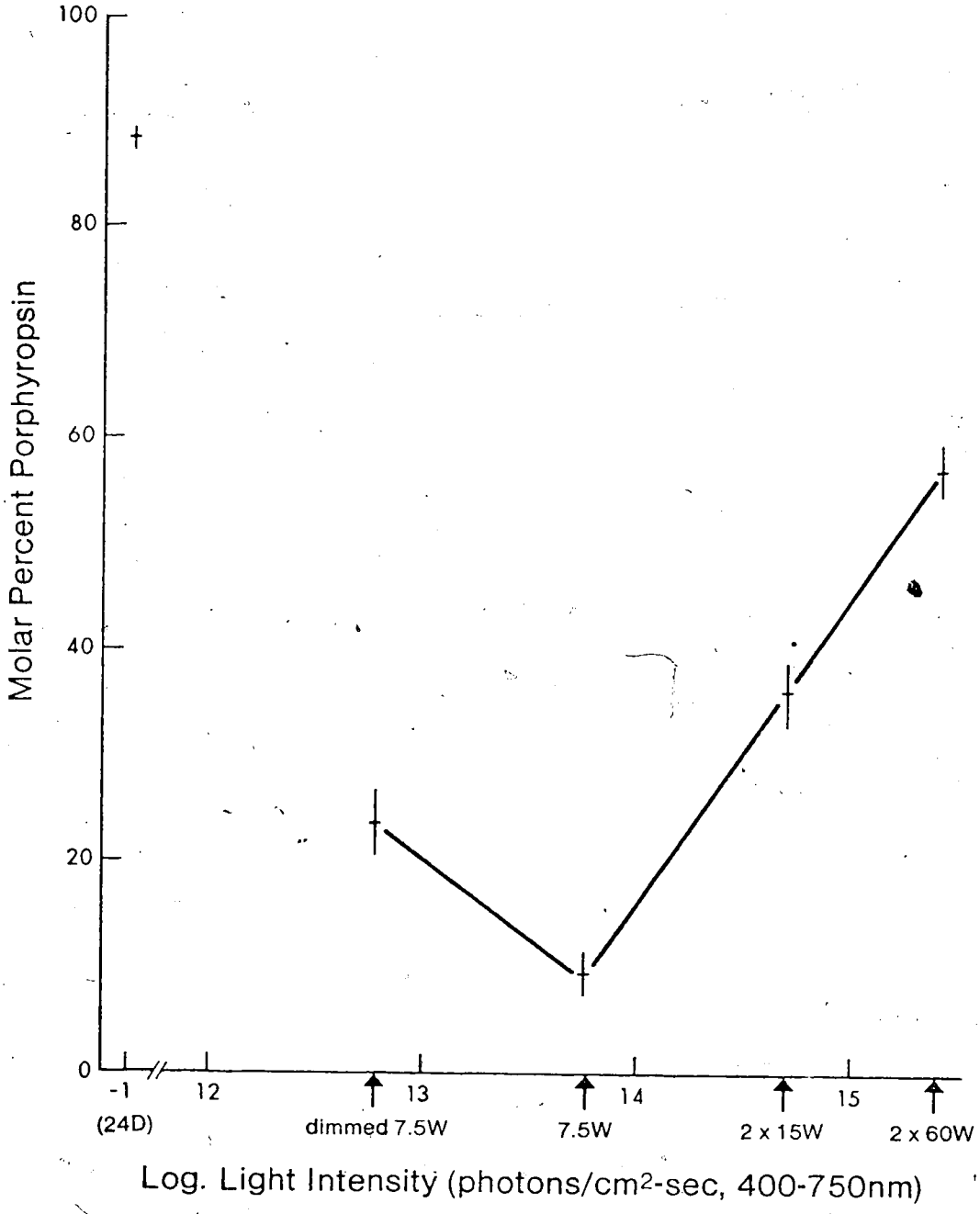
At a photoperiod of 16L/8D (at 30^0C), the increase or decrease of light intensity from 6.0×10^{13} photons/cm^2- sec, 400-750 nm (i.e. by a 7.5W light bulb) resulted in increases in the porphyropsin proportions (Fig. 4b). The light intensity provided by a 7.5W light bulb (at a photoperiod of 16L/8D and at 30^0C), therefore appears to be an optimum light intensity favoring rhodopsin. Above the light intensity of 6.0×10^{13} photons/cm^2-sec, 400-750 nm, the relationship of molar percent porphyropsin versus log photons/cm^2-sec, 400-750 nm appeared linear (Fig. 4b).

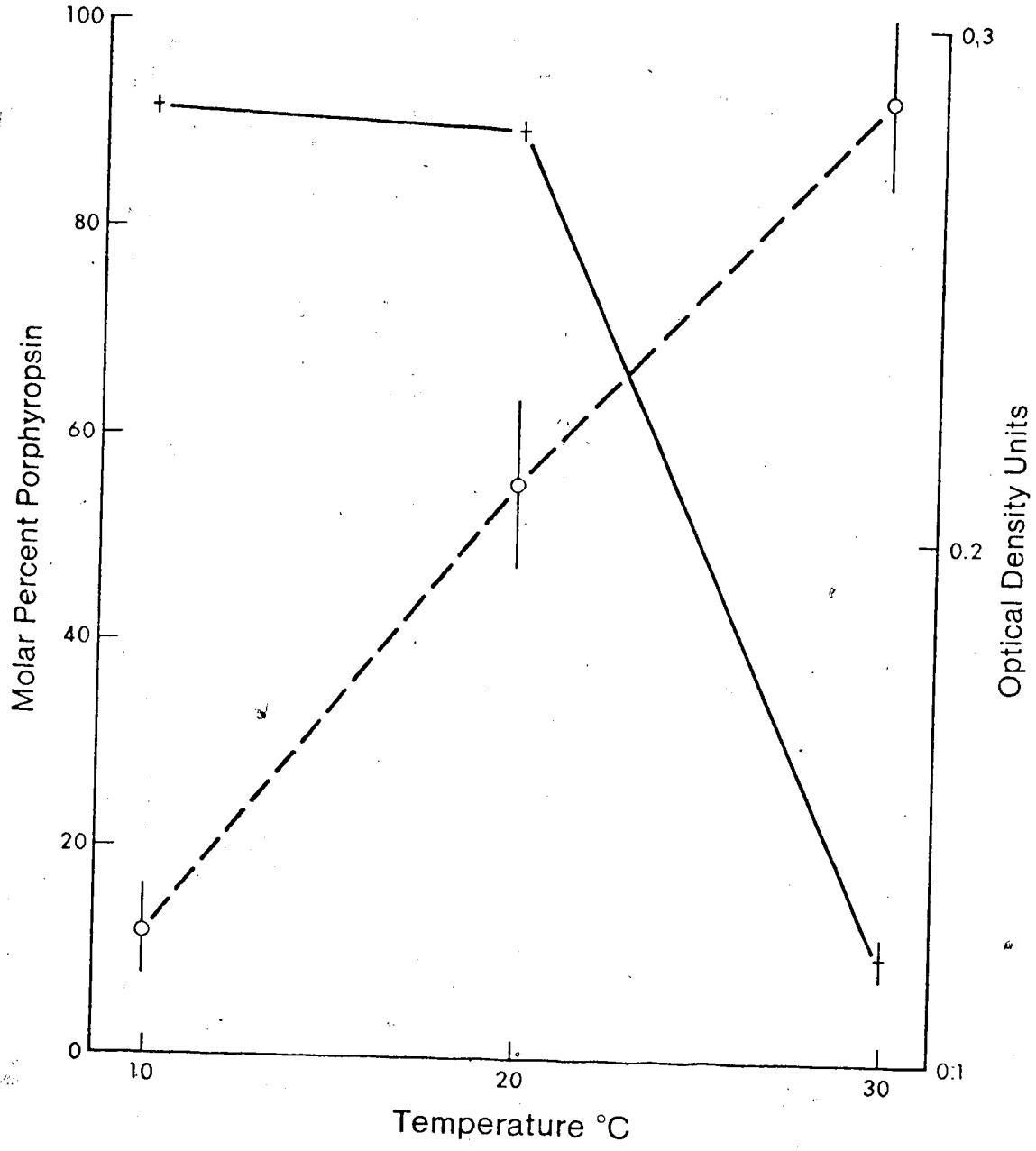
Under an intensity of 6.0×10^{13} photons/cm^2-sec (i.e. by a 7.5W light bulb) and at a photoperiod of 16L/8D, both 10^0C and 20^0C favored predominately porphyropsin whereas at 30^0C, rhodopsin predominated (Fig. 4c). During pigment analysis, it was noted that

Fig. 4 The effects of a) photoperiod b) light intensity and c) temperature on the visual pigment composition in goldfish.

Upon arrival from Grassyfork Fisheries, they had 93.3% porphyropsin (n=10). Thirteen fish each were placed into nine photoperiod, light intensity, temperature regimes (see Methods) and the visual pigments were analysed on the 50th day of acclimation. The means± 1 S.E. are indicated in the figure. All goldfish in a) were held at 30^{0}C under a 7.5W light bulb (6.0 X 10^{13} photons/cm^2-sec, 400-750 nm); those in b) were held in 30^{0}C at a photoperiod of 16L/8D; those in c) were held at a photoperiod of 16L/8D delivered by a 7.5W light bulb. The dashed line in c) represents the change in optical density of visual pigment extracts from individual fish held at different temperatures.


100
80
60
40
20
0
Molar Percent Porphyropsin
24L
16L/8D
8L/16D
24D
Photoperiod


100
80
60
40
20
0
Molar Percent Porphyropsin
-1
(24D)
12
13
dimmed 7.5W
14
7.5W
15
2 x 15W
2 x 60W
Log. Light Intensity (photons/cm2-sec, 400-750nm)


100
80
60
40
20
0
Molar Percent Porphyropsin
0.3
0.2
0.1
Optical Density Units
10
20
30
Temperature °C

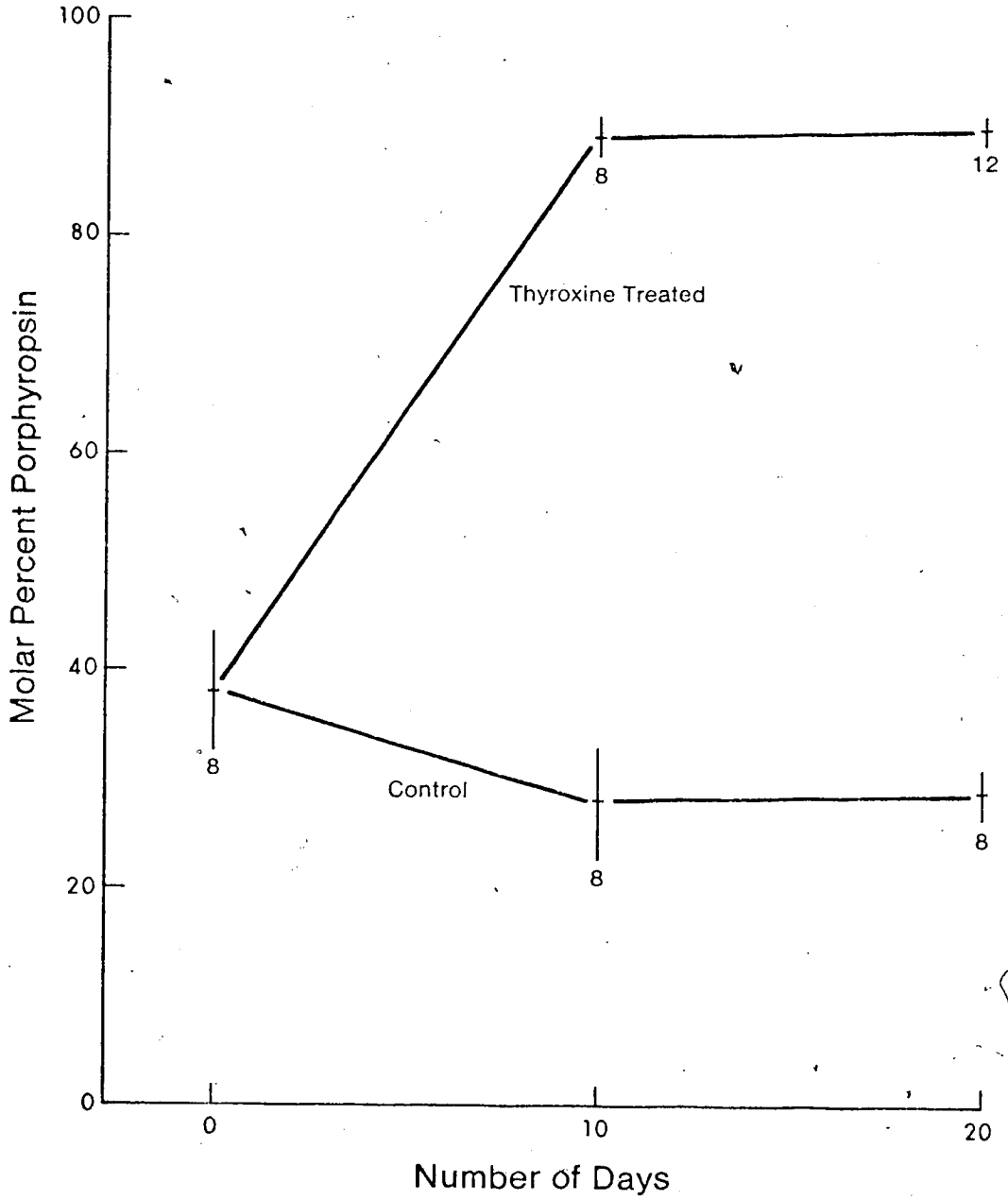
lower amounts of total visual pigment (based on absorbance units) were extracted from the lower temperature groups (Fig. 4c). Even though porphyropsin has a molar extinction coefficient about 75% of that of rhodopsin (see Methods), the lowest temperature group (i.e. 10^0C having predominately porphyropsin) possessed only about one third the amount of visual pigments in their retinas as that of the highest temperature group (i.e. 30^0C, having predominately rhodopsin). Thus this decrease cannot be attributed solely to an equimolar replacement of rhodopsin by prophyropsin but must be related to an absolute change in the total amount of visual pigment in the photoreceptor cells.

Effect of exogenous thyroxine on visual pigment composition (Ozark Goldfish)

Ozark goldfish exposed to a 7.5W bulb at a photoperiod of 16L/8D at 28^0C for 29 days also exhibited a decrease in the percentages of porphyropsin similar to Grassyfork fish (see Methods or legend of Fig. 5). Thyroxine treatment at a concentration of 100 µg L-thyroxine per liter tank water caused significant increases in the proportions of porphyropsin at both the 10^{th} and 20^{th} days of treatment (Fig. 5, Appendix 4) in comparison to controls. The relatively stable visual pigment compositions of the control groups suggest that in the goldfish a steady-state visual pigment composition (analgous to the steady-state visual pigment composition in trout) may also require 30-50 days to establish (see Chap II).

Fig. 5 Effect of exogenous thyroxine on the visual pigment composition in goldfish.

Goldfish were obtained from Hartz Mountain Pet Supplies (which obtained their goldfish only from Ozark Fisheries). After holding for 29 days at 28^0C under a 7.5W light bulb at a photoperiod of 16L/8D, their visual pigment composition changed from 86.6% porphyropsin (n=10, upon arrival) to 38.0% porphyropsin (n=8). Thyroxine treatment (L-thyroxine introduced to the tank at a concentration of 100 µg per liter tank water) commenced on the 30^{th} day after arrival (Day 0), with no change in the light and temperature regime. Mean ± 1 S.E. (sample size) are indicated in the Figure.


100
80
60
40
20
0
Molar Percent Porphyropsin
Thyroxine Treated
Control
8
12
8
8
8
0
10
20
Number of Days

Vitamin A composition in the pigment epithelium of goldfish

Although light, temperature (Fig. 4) and thyroxine (Fig. 5) treatments resulted in large changes in the visual pigment compositions in the retinas of the goldfish, the percentages of 3-dehydroretinol in the pigment epithelium of these fish remained high (more than 80%) (Fig. 6, Appendices 3 & 4). The molar porphyropsin percentages did not mirror the 3-dehydroretinol percentages in the pigment epithelium (Fig. 6), although there is a clear indication that lower 3-dehydroretinol proportions in the pigment epithelium were associated with rhodopsin-rich retinas (Fig. 6).

Long term conditioning of goldfish to a 15W bulb at a photoperiod of 16L/8D at 28^{0}C effectively lowered both porphyropsin ratios in the retinas and 3-dehyroretinol proportions in the pigment epithelium (Fig. 7, Appendix 5). This decrease in 3-dehydroretinol percentages appeared also to be directly related with time. Extrapolation of results suggests that the vitamin A composition in the pigment epithelium may match the visual pigment composition in the retina at approximately 600 days of acclimation under these light and temperature conditions (Fig. 7).

Fig. 6 The vitamin A composition in the saponified pigment epithelium in relation to the visual pigment composition in the retina in goldfish treated with light, temperature and thyroxine.

The data represent results from some 150 individuals used in Fig. 4 and 5 to test the effects of light, temperature and thyroxine. Filled circles represent means from thyroxine treated groups (Fig. 5) and open circles are means for others (Fig. 4a, b & c and controls of Fig. 5). Means± 1 S.E. are indicated in the Figure. Sample size was 13 per group unless otherwise indicated. The left insert represents the $SbCl_3$ absorbance spectrum for the extract of the pigment epithelium (78.8% 3-dehydroretinol) from a fish having rhodopsin rich retinas (90% rhodopsin). The right insert represents the $SbCl_3$ absorbance spectrum of the pigment epithelium (99.5% 3-dehydroretinol) from a fish having porphyropsin rich retinas (90% porphyropsin).

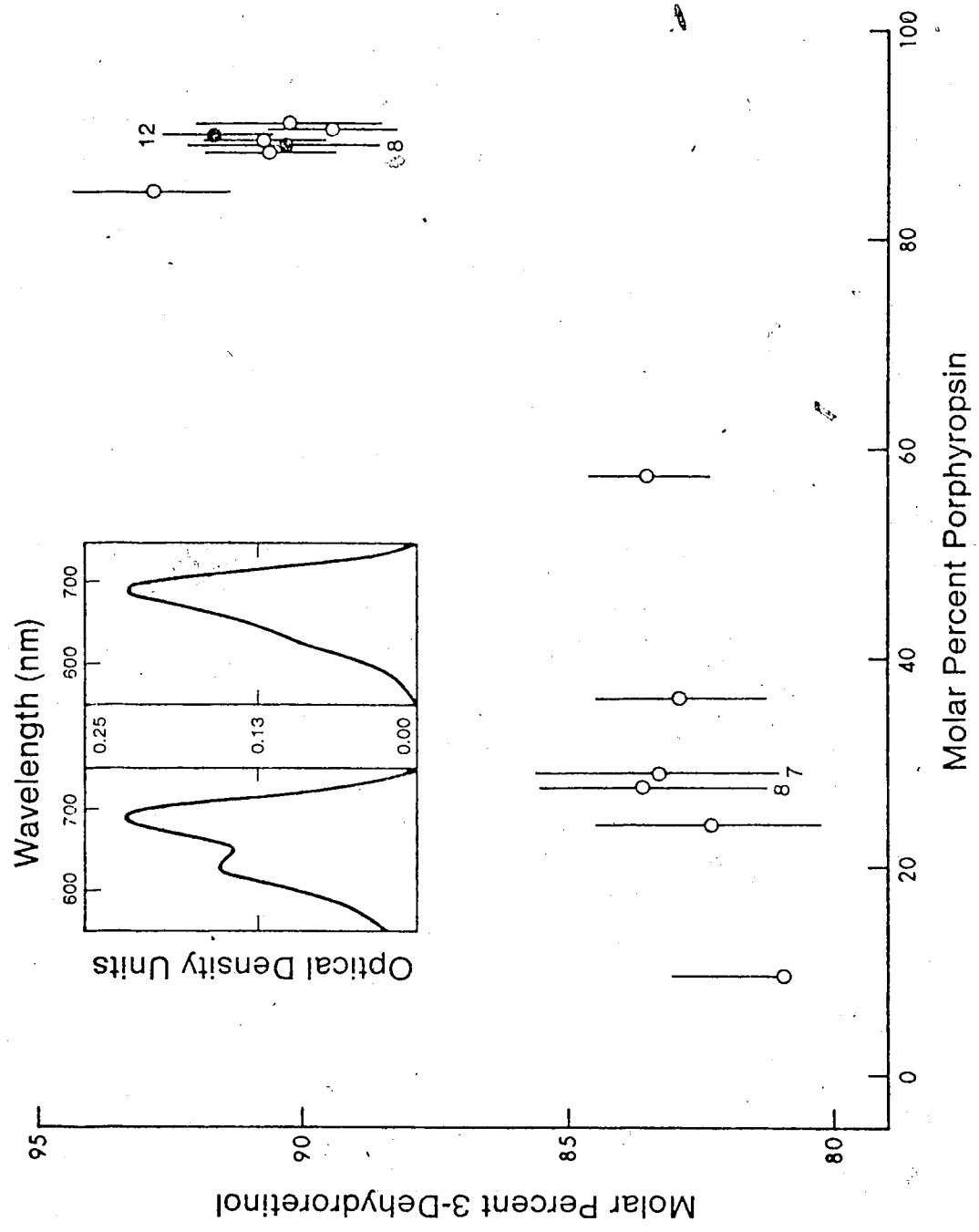
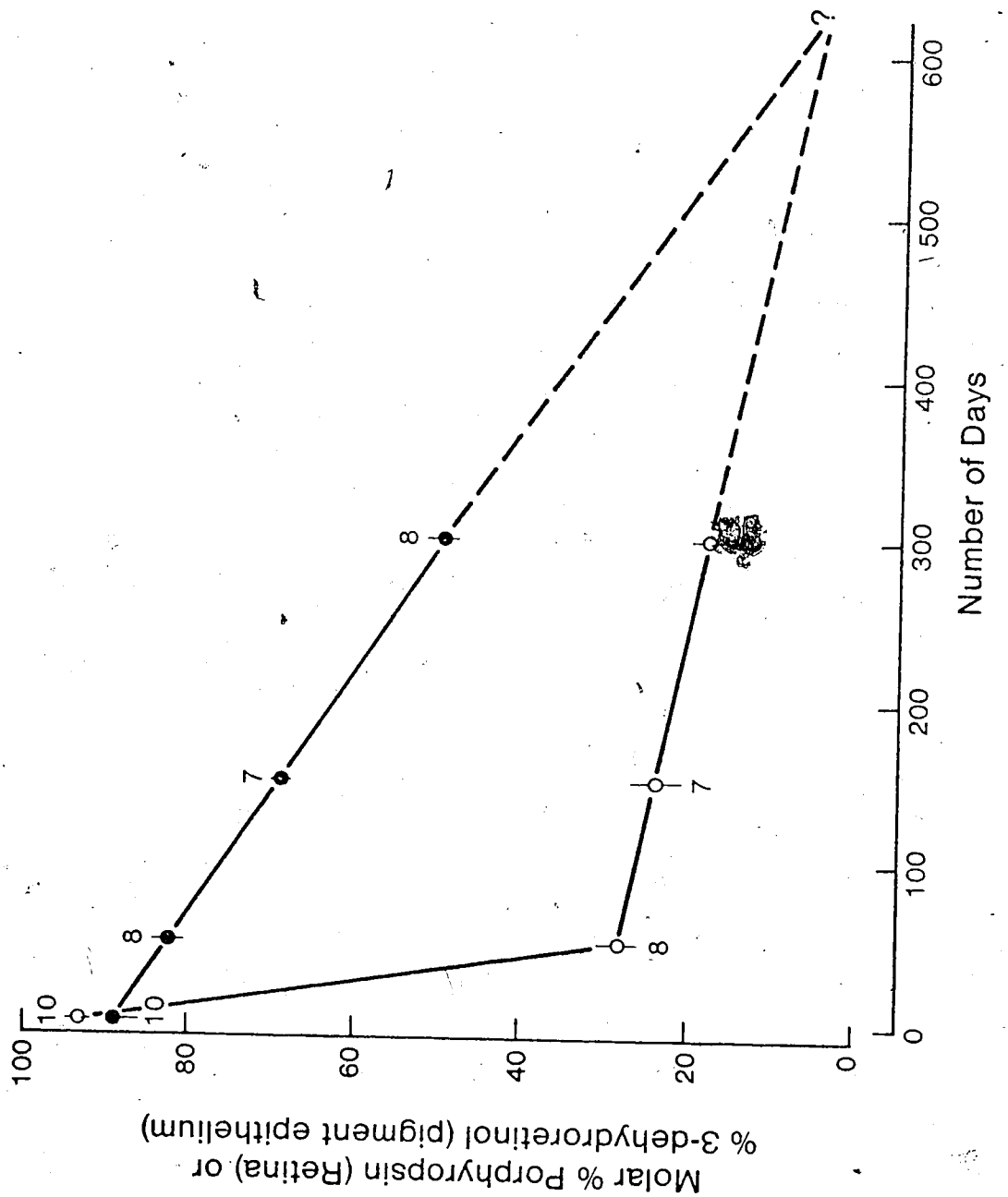

Molar Percent 3-Dehydroretinol
95
90
85
80
0
20
40
60
80
100
Molar Percent Porphyropsin
12
8
7
8
Optical Density Units
0.25
0.13
0.00
Wavelength (nm)
600
700
600
700

Fig. 7 The effect of long term acclimation (favoring rhodopsin rich retinas) on the vitamin A composition in the pigment epithelium of the goldfish.

Grassyfork goldfish were held in the holding tank (15W, 16L/8D, 28^{0}C) for a long period of up to 300 days. Means ± 1 S.E. (sample size) from serial samplings are indicated in the Figure. Open circles represent visual pigment composition in the retina and filled circles represent vitamin A composition in the saponified pigment epithelium. Dashed lines are extrapolations.


Molar % Porphyropsin (Retina) or
% 3-dehydroretinol (pigment epithelium)
100
80
60
40
20
0
0
100
200
300
400
500
600
Number of Days
10
10
8
7
8
8
7
8
?

Discussion

This study is the first to demonstrate the presence of goldfish rhodopsin which is not restricted to a population from a single supplier (Grassyfork Fisheries), as I have also detected it in goldfish from Ozark Fisheries. Although previous studies (Schwanzara, 1967; Bridges, 1973, 1975) did not report its presence, it is not totally surprising that goldfish can have rhodopsin in as much as the liver possesses a considerable quantity of retinol (Hata, Hata and Onishi, 1973; Bridges, 1975; the present study shows that the liver 3-dehydroretinol proportions did not bear any correlations to the porphyropsin proportions in the retinas, see Appendices 3, 4, and 5). It now seems evident that the formation of relatively large proportions of rhodopsin in goldfish requires the specific light and temperature regimes employed, whereas the previous studies did not use goldfish exposed to the required light and temperature combination (especially the higher temperature). The ability to form rhodopsin, in a species previously considered as having only porphyropsin, suggests that the temperature and light conditions should be considered in studies where there is uncertainty whether a single type of scotopic visual pigment (rhodopsin or porphyropsin) may exist. This would seem to be expecially relevant when the appropriate carotenoid presursors, retinol and 3-dehydroretinol are both known to be present in the body (e.g. in the liver). An example is the unresolved case of the visual pigments of the platanna, *Xenopus laevis*. Dartnall (1956) found small amounts of rhodopsin in addition to porphyropsin, in adult platannas which agreed with Wald's (1956) report of small amounts of

vitamin A_1 in addition to vitamin A_2, in the bleached retinas and the pigment epithelium. Subsequently, Crescitelli (1973) noted the presence of about 40% rhodopsin in tadpoles and nearly pure porphyropsin in adults, suggesting a changeover of visual pigments accompanying metamorphosis but in a direction the reverse of that for other anurans (for review see Crescitelli, 1972). Bridges (1974) and Bridges, Hollyfield, Witkovsky and Gallin (1977) next reported that both the tadpole and adult stages of the platanna have only porphyropsin but that the adult liver contained 62% retinol (Bridges *et. al.*, 1977). Is it possible that the light and temperature condition to which Crescitelli exposed his tadpoles favored the formation of some rhodopsin whereas Bridges' experiments did not? It is likely that certain species known to possess the appropriate carotenoid precursors, retinol and 3-dehydroretinol, may have both rhodopsin and porphyropsin if the light and temperature regimes are manipulated in a specific way whereas they may otherwise have only a single type of scotopic visual pigment.

The initial discovery of the labile nature of scotopic visual pigments was made on the rudd by Dartnall *et. al.*, (1961). From this and subsequent studies (Bridges and Yoshikami, 1970a; Muntz and Northmore, 1971) there is the general conclusion that the rudd responds to decreasing illumination (including constant darkness) by increasing the proportion of porphyropsin and to increasing illumination (including constant light) by increasing the proportion of rhodopsin. A similar pattern has also been observed in the pike killifish, golden shiner, and common shiner (Bridges, 1964, 1965; Allen and

McFarland, 1973; McFarland and Allen, 1977). Allen (1971) however reported that in the redside shiner, light favors porphyropsin and darkness favors rhodopsin. In addition, on testing the effect of photoperiod and light intensity, Allen concluded that for a given light intensity, a 12L/12D photoperiod was the most effective in promoting higher proportions of porphyropsin and that higher light intensities for a given photoperiod also favored porphyropsin. A similar effect of light favoring porphyropsin and darkness rhodopsin, occurs in rainbow trout and brook trout (Allen *et. al.*, 1973; McFarland and Allen, 1977; Cristy, 1976). On the basis of the apparent difference in the response to light, McFarland and Allen (1977) concluded that "fishes whose paired pigments are influenced by light divide into two distinct groups: those that increase rhodopsin in light ('rudd' response) and those that increase porphyropsin in light ('antirudd' response)" (also see Munz and McFarland, 1977). These two categories now seem inappropriate because they confuse the question of the response to constant darkness and light (not necessarily photoperiod or intensity related) with the response to different photoperiods and light intensities. For example, the visual pigment composition of the goldfish in the present study was strongly influenced by light; however, those in 24D, 8L/16D and 24L maintained high proportions of porphyropsin, thus showing both the 'rudd' response (to 24D) and the 'antirudd' response (to 8L/16D and 24L) whereas those in 16L/8D shifted to predominately rhodopsin ('rudd' response). Moreover, goldfish at the highest and lowest intensities had more porphyropsin than ones at an intermediate intensity giving on the one hand a 'rudd' response (to low intensity)

and on the other and 'antirudd' response (to high intensity). Systematic investigations of the responses of the rudd visual pigment system to photoperiod and light intensity have not been done and until such time it is perhaps premature to use the general response of this species to light and darkness as the model to which all other paired-pigment species are compared. Also, one cannot divorce the species' response to a given set of light conditions from the possible effect of temperature.

It is tempting to speculate that the increase in the proportion of rhodopsin in goldfish held in a 16L/8D photoperiod may be related to disc addition and disc shedding in the rod outer segments. Besharse, Hollyfield and Rayborn (1977) concluded that the maximum rate of disc addition and shedding in the rod outer segment of *Xenopus* tadpoles occurs in a light-dark cycle (12L/12D) in comparison to constant light or darkness. It is possible that the newly synthesized opsin, incorporated into the base of the rod outer segment during disc addition, may preferentially combine with 11-cis retinal to form rhodopsin. Loew and Dartnall (1976), using microspectrophotometry, found that the base of the rod outer segment of rudd, transferred from 12L/12D to continuous light for four weeks, was richer in rhodopsin than the apical end. This suggests that the increase in rhodopsin (which occurs in response to an increased light period in the rudd) results primarily from the formation of new visual pigment during disc addition to the base of the rod outer segment. Whether light intensity, in addition to photoperiod, may also have an effect on the rate of disc renewal is not presently known.

Goldfish, which maintained high proportions of porphyropsin at 10^0C and 20^0C and shifted to predominately rhodopsin at 30^0C (in a 16L/8D cycle), exhibit a response to temperature that has been observed in other species, notably the golden shiner (Allen and McFarland, 1973), common shiner (McFarland and Allen, 1977), and rainbow trout (Cristy, 1976; Tsin and Beatty, 1977; McFarland and Allen, 1977). It is evident however that higher temperatures alone will not promote a shift to rhodopsin as fish held at 30^0C in 24L, 8L/16D and 24D maintained high levels of porphyropsin (Fig. 4a). Whether other temperatures at these three light conditions would have been effective is uncertain; however it is tempting to suggest that temperatures higher than 30^0C might be, providing the temperature tolerance of the fish is not exceeded. Clearly temperature photoperiod and light intensity interact in altering visual pigment composition.

There is no apparent answer regarding the relationship between temperature and significant differences in the absorbance values of the visual pigment extracts (Fig. 4c). A change in the amount of visual pigment could result if there is an increase or a decrease (Bridges, Hollyfield, Besharse and Rayborn, 1976) in the length of the rod outer segment in response to temperature. At the lower temperature (10^0C), the rate of disc addition at the base of the rod outer segment may be reduced as Young (1967) demonstrated in *Rana pipiens*. This reduction could alter the balance between disc shedding from the tip of the rod outer segment and disc addition resulting in a decrease in the length of rod outer segment. More recently, O'Day and Young (1979) have reported that the length of the rod outer segment of goldfish held at 6^0C for

two months was 72% of the length of those from goldfish at 24°C. In addition, they also noted some degeneration of rod outer segment in the 6°C fish. The combination of shorter rod outer segment and degeneration would explain the reduction in the amount of visual pigment that is extracted from goldfish held at 10°C. On the other hand, the larger amount of visual pigment in extracts from goldfish held at 30°C could result from an increase in the length of the rod outer segment representing a reduction in the rate of disc shedding and/or an increase in the rate of disc addition. There is no current evidence, however, that this occurs in any species in response to high temperatures. Alternatively, lower temperatures may reduce the amount of visual pigment that is regenerated during dark adaptation.

The treatment of goldfish, pretreated with 16L/8D and 28°C in order to bring about a rhodopsin dominated retina, with exogenous thyroxine resulted in a shift back to predominately porphyropsin. This suggests that thyroxine can reverse the temperature-light induced increase in rhodopsin and implies that a neuroendocrine mechanism may play an important role in controlling visual pigment composition (see Beatty, 1975a). Similar effects of thyroxine have been found in other species (Beatty, 1969a; Allen, 1971; Jacquest and Beatty, 1972; Cristy, 1974; McFarland and Allen, 1977).

There is a general assumption, based on a relatively few studies (Wald, 1939; Bridges and Yoshikami, 1970b; Reuter *et. al.*, 1971; Bridges, 1973, 1975; also see McFarland and Allen, 1977) that the proportions of vitamin A_1 (or retinol) and vitamin A_2 (or 3-dehydroretinol) in the pigment epithelium are similar to those of

rhodopsin and porphyropsin in the retina. The consequence of this is a visual pigment composition that is dependent on the availability of the two prosthetic groups in the pigment epithelium (Bridges, 1972) and from this there is the implication that changes in the ratio of rhodopsin to porphyropsin are preceeded by appropriate changes in the ratio of retinol to 3-dehydroretinol in the pigment epithelium. In the goldfish which has relatively large amounts of carotenoid in the pigment epithelium (41 moles of 3-dehydroretinol per mole of porphyropsin, Bridges, 1975), my results show that the pigment epithelium contained high percentages of 3-dehydroretinol (more than 80%, Fig. 6) even when the retina contained predominately rhodopsin. Thus a shift from a porphyropsin to a rhodopsin rich retina is not dependent on a concomitant increase in the proportion of retinol in the pigment epithelium.

Long term acclimation of goldfish favoring large proportions of rhodopsin will decrease the 3-dehydroretinol proportions in the pigment epithelium (Fig. 7). This suggests that the degree of 'resemblance' between values of 3-dehydroretinol percentages (in the pigment epithelium) and porphyropsin percentages (in the retina) is dependent on the length of acclimation of the animal. The earlier hypothesis (Bridges and Yoshikami, 1970b; Reuter *et. al.*, 1971) that visual pigment composition in the retina is regulated by the relative availability of vitamins A in the pigment epithelium, therefore needs to be modified.

In conclusion, the goldfish represents yet another species with paired visual pigments, one whose visual pigment system responds to photoperiod, light intensity and temperature and should prove to be

very useful in further comparative studies on this interesting phenomenon. It also seems evident that one should be careful in using the overly general terms of 'rudd' and 'anti-rudd' response to categorize the more complex responses of a species visual pigment system to light (photoperiod and intensity) and temperature.

CHAPTER IV

VISUAL PIGMENTS AND VITAMINS A IN BULLFROGS AND LEOPARD FROGS

Introduction

The labile nature of visual pigments in paired pigment animals is not restricted to fishes because changes in visual pigments have also been reported in tadpoles of certain amphibians. For example, Bridges (1970, 1974) reported that light, in comparison to darkness, favored higher proportions of porphyropsin in bullfrog tadpoles *(Rana catesbeiana)*, leopard frog tadpoles *(Rana pipiens)* and bronze frog tadpoles *(Rana clamitans)* (Bridges, 1970, 1974). Lower water temperature also seemed to favor more porphyropsin in the bullfrog tadpoles held in darkness (Bridges, 1974). Whether light and temperature would also affect the visual pigment composition in any adult *Rana* species, however is not known at present.

In an earlier report, Reuter, White and Wald (1971) revealed that winter collected adult bullfrogs possessed up to 40% porphyropsin in their retinas, with all of the porphyropsin segregated in the dorsal part. This was in contradiction to previous reports by Wald (1935-6, 37-8, 45-6), Crescitelli (1958) and Wilt (1959) that only rhodopsin or retinal based visual pigment(s) occurred in adult bullfrog retinas. Bridges, (1974) also confirmed that bullfrogs have porphyropsin separated into the dorsal (or superior) segment of the retina, as pointed out by Reuter *et. al.*, (1971). Bownds,

however, claimed that porphyropsin proportions in bullfrog retinas did not exceed 15% during several years of sampling (see discussion of Bownds *et. al.*, 1974). Could the disagreement in reports by different investigators be the result of light and temperature influences on the photopigment composition in the adult bullfrog?

This study shows that adult bullfrogs can also be induced to alter their visual pigment composition in the retina by certain treatments with light and temperature. The change of visual pigment composition in the retina as a whole, from approximately 50% porphyropsin to almost pure rhodopsin is the result of an almost complete replacement of porphyropsin by rhodopsin in the dorsal retina. The ventral retina remains rhodopsin rich despite the significant light and temperature influence on the dorsal retina. During the course of this study, it was also noted that bullfrog rhodopsin has an absorbance maximum of 499 nm, instead of 500-503 reported by earlier investigators (Wald, 1935-6; Crescitelli, 1958; Reuter *et. al.*, 1971). A parallel study on the visual pigments of the leopard frog *(Rana pipiens)* showed that similar light and temperatures (see Methods) did not change the visual pigment composition of their retinas which contained only rhodopsin. This agrees with Bridges' finding (Bridges, 1974) that bullfrogs may constitute an exception amongst many frogs (*Rana & Hyla* spp.) in possessing porphyropsin in their adult stage.

Comparison between the vitamin A proportion in the pigment epithelium[1] and the visual pigment composition in the retina of the

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[1] The pigment epithelium of the bullfrog was not saponified in this

bullfrog (leopard frogs do not have sufficient vitamin A in their pigment epithelium for analysis) indicated that in most cases, the vitamin A_2 proportion resembled the porphyropsin proportion. However exceptions were found in rhodopsin dominated dorsal retinas and in dorsal retinas from frogs kept under constant darkness. In both instances, vitamin A_2 proportions in the pigment epithelium were significantly higher than porphyropsin proportions in the retinas. These, together with earlier results from the goldfish (Chapter III, see also Discussion in the present Chapter) suggest that the relative proportion of vitamin A_1 and vitamin A_2 in the pigment epithelium does not determine the visual pigment composition in the retina.

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study because saponification (and subsequent washing and reconstitution) may reduce the vitamin A recovery from the bullfrog pigment epithelium which possesses only small amounts of vitamin A_1 and A_2.

Materials and Methods

Light and temperature environments

The controlled light and temperature environments have been described in detail earlier (Tsin, 1976; see also Chapter III). Dechlorinated warm (30^0C) or cold (5^0C) water continuously flowed into the tank to maintain the desired water temperatures (see Tsin, 1976). Light intensities and photoperiods were attained by employing tungsten light bulbs (7.5W or two 60W) in connection to automatic timers. The water level inside the tank was approximately 7 cm deep so that bullfrogs were mostly submerged. Leopard frogs (a more terrestrial species) were only partly submerged (water levels about 2-3 cm deep). Air temperature closely resembled the water temperature. Animals were fed with house crickets and no mortality occurred during the experiment.

Analysis of visual pigment and vitamin A

The eyes of the bullfrog were removed (under dim red light) with a pair of enucleation scissors. Each eye was opened by cutting around the cornea and the lens was then removed. The posterior eye cup was divided into a dorsal and a ventral segment, the line of sectioning being about 1 mm dorsal to the optic nerve stalk (for an illustration, see Fig. 4 of Reuter *et. al.*, 1971). The dorsal and ventral retinas were then removed from each segment and stored separately in 4% potassium aluminum sulfate at -20^0C. The dorsal and ventral pigment epithelia were also removed and stored in phosphate buffer (pH=7.3) for later vitamin A analysis.

Visual pigment extraction (0.5 ml of 2% digitonin) and analysis (Beatty, 1969) were performed on individual bullfrog retinal segments. About 80% of all retinal extracts (groups A-H, see later paragraphs for details) were analysed by partial bleaching experiments, employing the bleaching protocol of : 1 min. deep red light (675 nm), 4 min. red light (660 nm) and 8 min. orange light (610 nm) exposures. The remainder of the retinal extracts (groups I-L) were analysed by bleaching experiments consisting of one single ten min. exposure to orange light. Difference spectra derived from partial bleaching experiments performed on porphyropsin-rich and rhodopsin-rich retinal extracts in combinations with standard visual pigment nomograms (Munz and Swanzara, 1967; Wyszecki and Stiles, 1967), indicated that bullfrogs possess a porphyropsin (λmax=522 nm) and a rhodopsin (λmax=499 nm). The rhodopsin absorbance maximum was slightly lower than the 500-503 nm previously reported for bullfrog rhodopsin (Wald, 1937-8; Crescitelli, 1958 and Reuter *et. al.*, 1971) and this discrepancy will be discussed later. The visual pigment composition (molar percent porphyropsin) in each extract was derived following standard procedures (see Tsin, 1976, Appendix 2).

The vitamins A_1 and A_2 were extracted from freeze-dried bullfrog pigment epithelium. The left and right dorsal (or ventral) pigment epithelia from a single frog were pooled and extracted with two 3 ml portions of petroleum ether (B.P. 37.4-58.8^{0}C) under dim red light (Kodak Safelight with a Wratten Series 2 Filter). The pooled petroleum ether was then dried under a stream of oxygen-free dry nitrogen and the residue redissolved in 1 ml chloroform (under a 40W red bulb illumination). The liver samples were freeze-dried,

saponified, extracted with ether, dried under nitrogen and redissolve in chloroform.

The reconstituted vitamin A in chloroform was added (10-100µl) to 1 ml saturated antimony trichloride solution for the Carr-Price colorimetric determination for vitamins A (Hubbard, Brown and Bownds, 1971). The vitamin A_2 proportions were calculated from the absorbance spectra using simultaneous equations previously developed (Hubbard *et. al.*, 1971).

The left and right retinas of the leopard frog were pooled to prepare the 0.5 ml 2% digitonin extract. The pigment epithelium of leopard frog did not have sufficient storage vitamin A for analysis. The liver vitamin A was analysed using the same method mentioned above for the bullfrog.

Effects of light intensity, photoperiod and temperature on the visual pigments and vitamins A in the bullfrog

Adult bullfrogs, *Rana catesbeiana*, were obtained from the Canadian Breeding Farm and Laboratories Ltd. in Quebec (Canada). According to the supplier, these frogs were field-collected and held in outdoor ponds before delivery in October, 1978. Upon arrival they had 38.0% porphyropsin (n=2) in their whole retinas (mean body weight: 192 gm).

A total of 12 light-temperature conditions were tested in this study. Upon arrival, 16 frogs were equally divided into 8 groups and conditioned for 40 days (October-December, 1978, groups A-H):

18L/6D				6L/18D			
7.5W		2X60W		7.5W		2X60W	
10^0C	30^0C	10^0C	30^0C	10^0C	30^0C	10^0C	30^0C
A	B	C	D	E	F	G	H

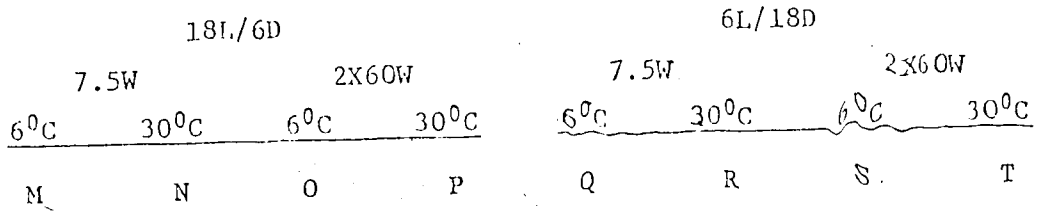
The two different light intensities, as measured at water surface employing the remote probe of a spectroradiometer (between 400-750 nm) were: 6.0×10^{13} photons/cm^2-sec (7.5W) and 2.4×10^{15} photons/cm^2-sec (two 60W bulbs).

Four additional groups (two frogs per group, groups I-L) were likewise subjected to 40 days of treatment at a later date (December 1978-January 1979). Before they were transferred from the holding tank (18L/6D, 7.5W, 15^0C) to the experimental tanks, they had 30.0% porphyropsin (n=1) in their whole retinas. These additional conditions were:

18L/6D	24L	12L/12D	24D
2X60W	2X60W	2X60W	
20^0C	10^0C	10^0C	10^0C
I	J	K	L

Effects of light intensity, photoperiod and temperature on the visual pigments in the leopard frog

Adult leopard frogs, *Rana pipiens*, were obtained from Narsco Educational Materials, Ltd. in Guelph, Ontario. Upon arrival in the fall, 1978, they (mean body weight: 50.8 gm) were subjected to 30 days of different light and temperature treatments (about 3-4 frogs per group, groups M-T). These conditions were:



Partial bleaching experiments showed that all frogs possessed only rhodopsin (λmax=502 nm) irrespective of the environmental regimes (Appendix 6). Their liver possessed predominately retinol (Appendix 6). The subsequent section (Results) therefore will present results from the bullfrog only (Appendix 7).

Results

Light and temperature effects on the visual pigments in the bullfrog

After 40 days, bullfrog retinas may possess predominately rhodopsin (see whole retinas of groups B, D, F, H, Table 1) or up to 50% porphyropsin (group C, Table 1), (see also Appendix 7). This change in photopigment proportions is primarily the result of changes in the visual pigment composition in the dorsal retinas in response to light and temperature treatment (Table 1). A replacement of porphyropsin by rhodopsin in the dorsal retinas is evident in certain groups (compare groups C & D; G & H, Table 1). The ventral retinas, however, always remained rhodopsin rich (Table 1).

Lower water temperatures at each photoperiod and light intensity induced higher porphyropsin porportions in all dorsal and ventral retinas (compare groups A & B, C & D for dorsal and ventral retinas, groups E & F, G & H for dorsal retinas). Higher light intensity also favored higher porphyropsin proportions (compare groups A & C, dorsal and ventral retinas, and groups E & G, dorsal retinas, Table 1). The effect of photoperiod can be revealed by comparing frogs held in the lower temperature (10^0C; since the higher temperature, 30^0C, tends to favor almost exclusively rhodopsin in all cases). Longer photoperiod consistently favored higher porphyropsin proportions in all these groups (compare groups A & E, C & G, dorsal and ventral retinas, Table 1).

The effect of photoperiod is further substantiated when results of the two sets of experiments are considered (groups C & G from

Table 1. Visual pigment composition of dorsal, ventral and whole retinas of the adult bullfrog, *Rana catesbeiana*, kept under different light-temperature regimes for 40 days.

The porphyropsin percentages (for details, see Appendix 7) of the two frogs per group and their means are presented in the table. Each value of the dorsal (or ventral) retinas was obtained by averaging the visual pigment compositions of left dorsal (or ventral) and right dorsal (or ventral) retina of an individual. Each value of whole retinas was calculated from the weighed sum of dorsal and ventral retinas, i.e. taking into consideration the relative amounts of visual pigment in the dorsal and ventral segments. Note the effects of photoperiod, light intensity and temperature especially on the dorsal retinas of the bullfrog. These frogs had 38.0% porphyropsin (n=2) in their whole retinas when the experiment started, suggesting that they had upwards of 70% porphyropsin in the dorsal segments.

		18L/6D				6L/18D			
		7.5W		2X60W		7.5W		2X60W	
		10^0C	30^0C	10^0C	30^0C	10^0C	30^0C	10^0C	30^0C
		A	B	C	D	E	F	G	H
Dorsal		51.1	3.0	86.1	6.0	40.4	3.8	72.9	6.3
Retinas		52.5	6.0	78.4	7.3	36.4	7.3	69.9	7.3
	$\bar{x}$=	51.8	4.5	82.3	6.7	38.4	5.6	71.4	6.8
Ventral		12.7	1.9	16.8	1.9	6.2	3.0	12.0	4.9
Retinas		13.7	3.8	27.4	4.9	6.2	0.0	8.5	3.8
	$\bar{x}$=	13.2	2.9	22.1	3.4	6.2	1.5	10.3	4.4
Whole		28.8	2.2	48.0	4.0	18.7	3.7	35.8	5.4
Retinas		31.8	4.7	51.6	5.7	20.2	6.1	34.2	4.5
	$\bar{x}$=	30.3	3.5	49.8	4.9	19.4	4.9	35.0	4.9

Table 1 in addition to groups J, K and L). Figure 8a shows that in the dorsal retinas, constant darkness (group L, 46.9% porphyropsin) favored lower porphyropsin proportions in comparison to those held in light-dark cycles. Lengthening the photoperiod from 24D to 6L/18D and to 12L/12D resulted in increases of porphyropsin ratios (6L/18D, group G, 71.4% porphyropsin, 12L/12D, group K, 83,4% porphyropsin). Photoperiods longer than 12L/12D, however, did not appear to favor further increases in porphyropsin proportions (18L/6D, group C, 82.3% porphyropsin and 24L, group L, 84.4% porphyropsin). Ventral retinas, as have been noted before, remained always rhodopsin rich (less than 25% porphyropsin).

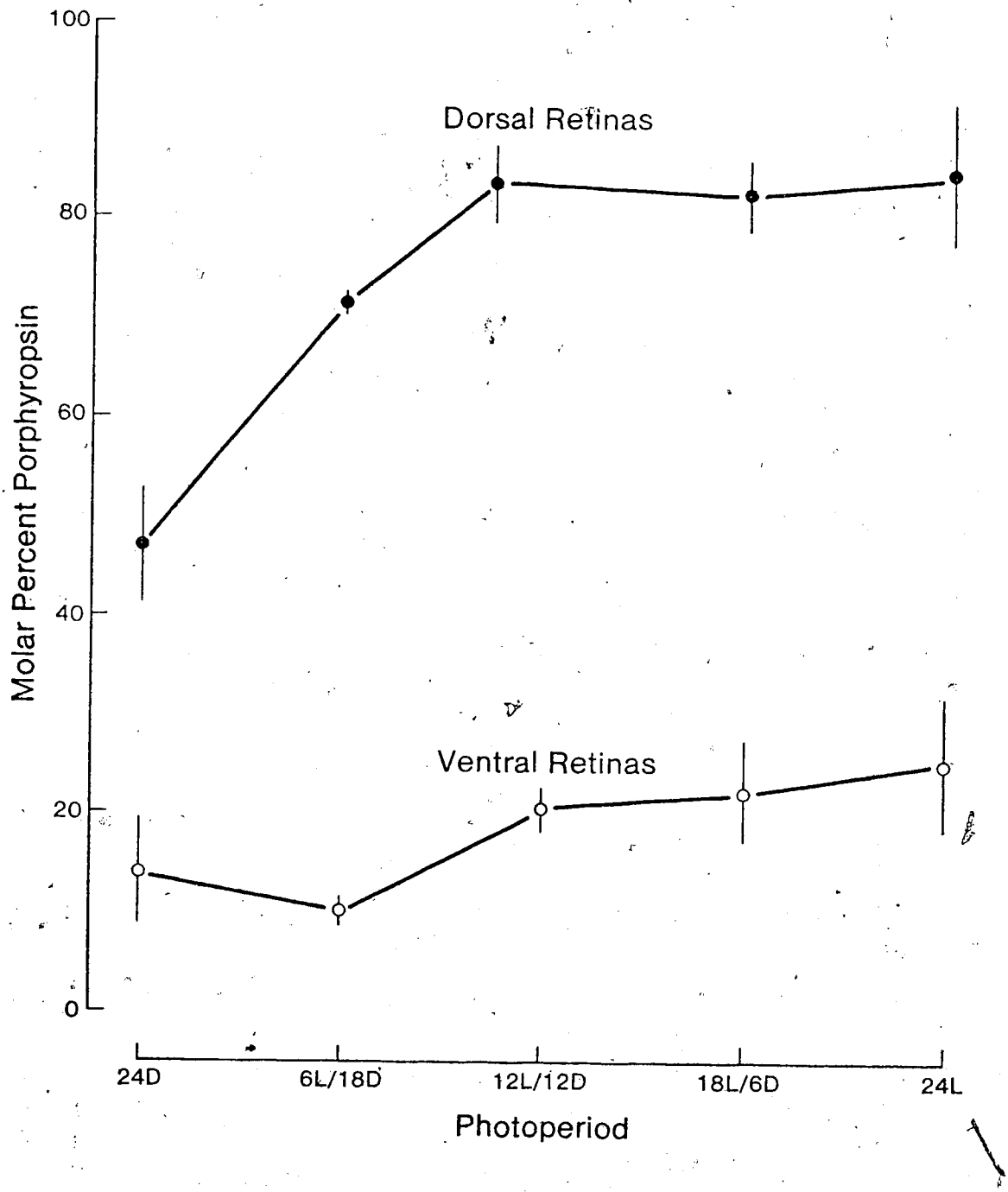
Analogous to the effect of photoperiod, temperature may, or may not be associated with a significant change in visual pigment composition of the dorsal retinas (Fig. 8b). Both 10^0C (group C, 82.3% porphyropsin) and 20^0C (group I, 79.3% porphyropsin), at the 18L/6D photoperiod and 2X60W intensity, favored higher proportions of porphyropsin, whereas frogs at 30^0C (group D, 6.7% porphyropsin) with the same photoperiod and light intensity, had predominately rhodopsin in the dorsal retinas (Fig. 8b). There is also an indication of a progressive increase of rhodopsin in the rhodopsin-rich ventral retina as temperature increased (Fig. 8b).

Visual pigment composition in relation to vitamin A composition in the pigment epithelium of the bullfrog

The vitamin A_2 proportion in the pigment epithelium in most instances resembled the porphyropsin proportion in the retina of the bullfrog (see Fig. 9 and Fig. 10). A rhodopsin-rich dorsal

Fig. 8. Visual pigment composition in the dorsal and the ventral retinas of adult bullfrog subjected to a) photoperiod and b) temperature treatments.

These animals were obtained in the winter of 1978 and subjected to the indicated conditions for 40 days before visual pigment analysis. Frogs in (a) were held under two 60 Watt light bulbs at 10^0C water. Frogs in (b) were held under a photoperiod of 18L/6D, delivered by two 60 Watt light bulbs. For details of the experiments, see Methods. The range and the group mean of two frogs per group are indicated in the Figures.


100
80
60
40
20
0
Molar Percent Porphyropsin
Dorsal Retinas
Ventral Retinas
24D
6L/18D
12L/12D
18L/6D
24L
Photoperiod

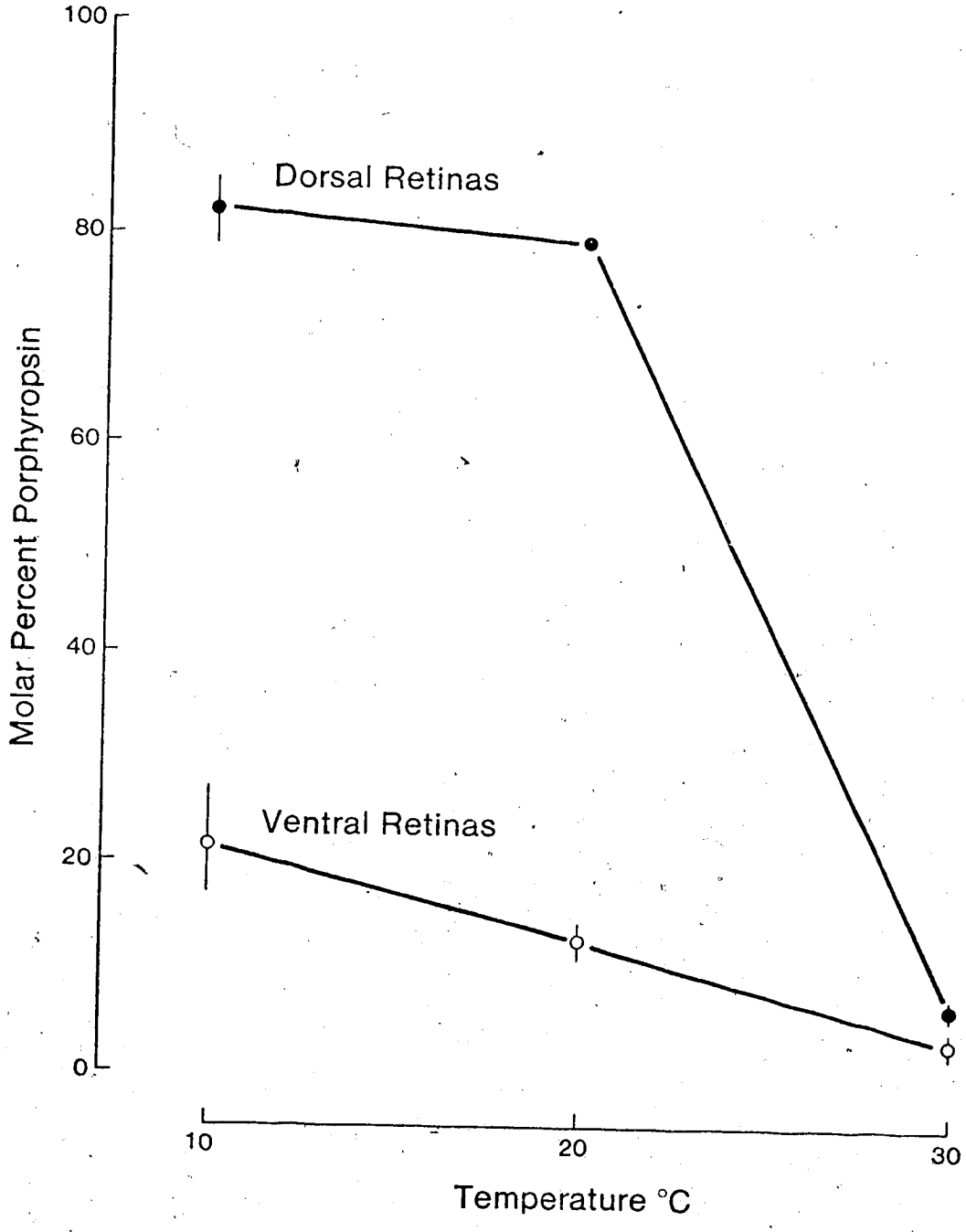

100
80
60
40
20
0
Molar Percent Porphyropsin
Dorsal Retinas
Ventral Retinas
10
20
30
Temperature °C

Fig. 9 Carr-Price spectra showing vitamin A compositions in the dorsal and the ventral pigment epithelium as well as in the liver of the adult bullfrog.

The Carr-Price colors were developed from the reactions between saturated antimony trichloride solution with vitamins A. Vitamin A_1 exhibits characteristic peak at 616 nm whereas vitamin A_2 shows peak color at 690 nm. A rhodopsin rich and a porphyropsin rich dorsal retina associated respectively with vitamin A_1 rich and vitamin A_2 rich pigment epithelium (Fig. 9a and 9c). A dorsal retina of similar rhodopsin-porphyropsin contributions had equal proportions of vitamin A_1 and vitamin A_2 (Fig. 9b). The rhodopsin rich ventral retina always associated with vitamin A_1 rich pigment epithelium (Fig. 9d) whereas the liver also had predominatley retinol (Fig. 9e).

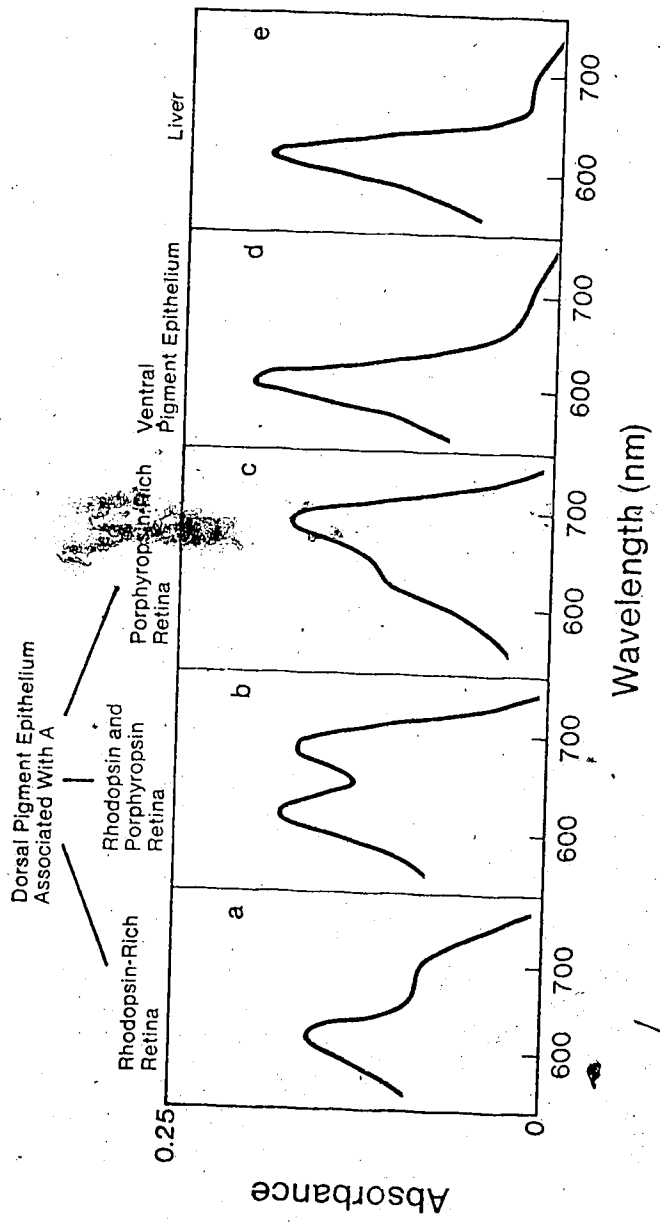
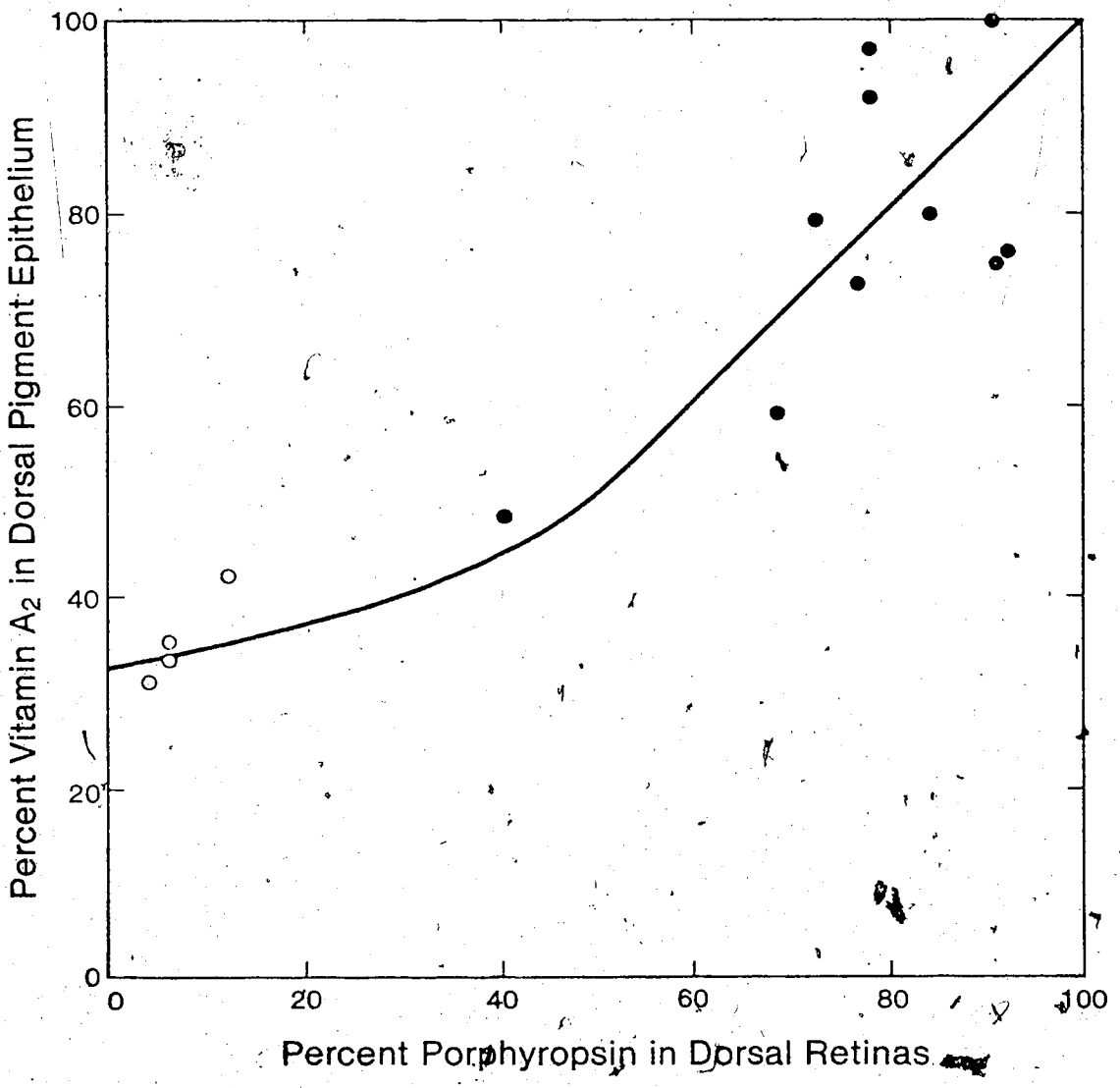

Absorbance
0.25
0
Wavelength (nm)
600
700
a
b
c
d
e
Rhodopsin-Rich Retina
Dorsal Pigment Epithelium Associated With A
Rhodopsin and Porphyropsin Retina
Porphyropsin-Rich Retina
Ventral Pigment Epithelium
Liver

Fig. 10 The visual pigment composition in the dorsal retina in relation to the vitamin A composition in the dorsal pigment epithelium in the adult bullfrog.

The different visual pigment compositions of the dorsal retinas were obtained by subjecting the bullfrog to different light-temperature conditions for 40 days (see Table 1, Fig. 8a and b, or Methods). Filled circles represent results from frog held under lower temperatures (10^0C, 20^0C) whereas open circles show results from a higher temperature (30^0C). The line was eye fitted.


Percent Vitamin A_2 in Dorsal Pigment Epithelium
100
80
60
40
20
0
0
20
40
60
80
100
Percent Porphyropsin in Dorsal Retinas

retina (6.0% porphyropsin) associated with a vitamin A_1-rich pigment epithelium (64.7% vitamin A_1, Fig. 9a) whereas a porphyropsin-rich dorsal retina (87.5% porphyropsin) associated with a vitamin A_2-rich epithelium (78.4% vitamin A_2, Fig. 9c). A dorsal retina having about equal amounts of the two pigments (40.4% porphyropsin) associated with a pigment epithelium having similar proportions of the two vitamins (48.2% vitamin A_2, Fig. 9b). The rhodopsin-dominated ventral retinas (more than 78% rhodopsin) always had a vitamin A_1-rich pigment epithelium (more than 80% vitamin A_1, Fig. 9d). The livers were also dominated by retinol (Fig. 9e).

The chloroform extracts of the (non-saponified) pigment epithelium contained certain carotenoids which may interfere with the vitamin A determinations using the Carr-Price Method. In this study, about 30% of vitamin A analyses were contaminated with these carotenoids. Therefore, in Figure 10, only the results from uncontaminated extracts of the dorsal pigment epithelium are presented. The animals held at 10^0C and 20^0C temperatures had vitamin A composition in their pigment epithelium similar to the visual pigment composition in their retinas (Fig. 10, [illegible]e line was eye fitted). However, those held in a warmer temperature with rhodopsin-rich retinas, had significantly higher vitamin A_2 proportions in their dorsal pigment epithelium in comparison to their porphyropsin proportions (25.9-42.4% vitamin A_2 in the pigment epithelium vs 3.8-7.3% porphyropsin in the retinas, open circles, Fig. 10). Moreover, the two frogs held in constant darkness at 10^0C (group L) also had higher percentages of vitamin A_2 in the dorsal pigment epithelium than the porphyropsin proportions in their dorsal retinas (Frog 1 had 70% vitamin A_2 vs 53% porphyropsin, Frog 2

had 87.1% vitamin A_2 vs 40.7% porphyropsin, these points are not included in Fig. 10).

Discussion

The absorbance maxima of bullfrog rhodopsin and porphyropsin

In 1937-8, Wald reported that bullfrogs possess a rhodopsin (λmax=500 nm) in their retinas (Wald, 1937-8). Later, he discovered that the metamorphosis of bullfrog tadpoles to adults involved a transition from retinas dominated by vitamin A_2 to retinas containing only vitamin A_1 (Wald, 1945-6). Subsequently, Crescitelli, using partial bleaching experiments, confirmed that bullfrog tadpoles had a mixture of rhodopsin (λmax=503 nm) and porphyropsin (λmax=525 nm) whereas the adult possessed only rhodopsin (λmax=503 nm) (Crescitelli, 1958; see also Crescitelli, 1972). Reuter *et. al.* (1971) pointed out that the adult bullfrog possessed both rhodopsin (λmax=502 nm) and porphyropsin (λmax=522 nm). In addition, they said 'the difference spectrum of the bullfrog tadpoles porphyropsin seems to be identical with that of the adult porphyropsin'. The present study, also employing partial bleaching experiments, suggests that adult bullfrogs possess rhodopsin of λmax=499 nm and porphyropsin of λmax=522 nm.

My results on the bullfrog rhodopsin and porphyropsin were obtained from partial bleaching experiments on rhodopsin-rich and porphyropsin rich retinal extracts. The difference spectra, obtained from bleaching with 610 nm and 675 nm light were used to estimate the the absorbance maxima for rhodopsin and porphyropsin respectively. These difference spectra were in full agreement with nomograms for a 499 nm rhodopsin (from the revised nomogram for rhodopsins by Dartnall, appearing in Wyszecki and Stiles, 1967) and a 522 nm

porphyropsin (from the nomogram for porphyropsins by Munz and Schwanzara, 1967). Moreover, when these absorbance maxima were applied to an established equation for the absorbance maxima of paired visual pigments in fishes (Dartnall and Lythgoe, 1965; Bridges, 1965b), a porphyropsin of λmax=522 nm is confirmed to be associated with a rhodopsin of λmax=499 nm.

Clearly, the absorbance maxima of both rhodopsin and porphyropsin of bullfrog tadpoles and adults have not been agreed upon in the literature and there is no apparent answer as to the cause of this discrepancy at present. Nevertheless, the small differences for the λmax values reported here and in other studies should not invalidate a comparison of the estimated relative proportions of the two pigments in various studies.

Visual pigment change induced by light and temperature

This study constitutes the first report on light and temperature induced changes in visual pigment composition in an adult amphibian. A light-dark cycle, at 10^0C, in comparison to continuous darkness, induces higher porphyropsin proportions in the bullfrog (Fig. 8a), a response similar to that exhibited by tadpoles of the bullfrog, leopard frog and the bronze frog (Bridges, 1970, 1974). Low temperature favors higher porphyropsin proportions in the adult (at 18L/6D) (Fig. 8b) as well as the tadpoles of the bullfrog (at 24D, Bridges, 1974).

A direct comparison of effect of photoperiod, light intensity and temperature on the proportions of rhodopsin and porphyropsin in the bullfrog and in paired visual pigment fishes may be difficult.

This is because different studies have employed different experimental protocols in addition to the different temperatures tolerated by different species. In spite of these limitations, it is quite surprising to find similarities in visual pigment response to these environmental parameters. For example, the photoperiod effect on the bullfrog porphyropsin proportions (Fig. 8a, frogs were held under 2X60W light bulb, at 10^0C water) appeared similar to the photoperiod effect on the visual pigment composition in rainbow trout (Fig. 2a, Chapter II, trout were held under two 20W fluorescent tubes, at various water temperatures). In both cases, higher photoperiods favored higher porphyropsin ratios. Another example is the similarity of the influence of temperature on the bullfrog visual pigments in the dorsal retinas (Fig. 8b. frogs were held under two 60W light bulbs at a photoperiod of 18L/6D) and the temperature effect on the goldfish visual pigments (Fig. 4c, Chapter III, goldfish were held under a 7.5W light bulb at 16L/8D). In both cases, 10-20^0C water favored high porphyropsin ratios whereas 30^0C favored predominately rhodopsin.

Although Reuter *et. al.* (1971) reported the presence of porphyropsin in adult bullfrog retinas, contrary to the conclusions of Wald (1935-6) and Crescitelli (1958), Reuter *et. al.* did not explain why winter frogs had more porphyropsin (30-40%) than summer frogs (5%) other than a possible seasonal effect. From this study, it appears that this seasonal difference could be attributed to an effect of temperature and/or light. Likewise it is possible that Wald (1935-6) and Crescitelli (1958) may have used bullfrogs which were acclimated to conditions favoring rhodopsin.

It is of interest to speculate why bullfrogs retain porphyropsin

in their adult phase. A parallel study on the visual pigments of the adult leopard frog (*Rana pipiens*) did not show any porphyropsin despite using similar light and temperature treatments (Appendix 6). This agrees with Bridges' (1974) results that adult bullfrogs constitute an exception amongst the several *Rana spp.* and *Hyla spp.* in having porphyropsin in their adult life. This may be because the adult bullfrog is a semi-aquatic species, preferring to stay in the water even after metamorphosis. As Reuter *et. al.* (1971) have pointed out, the ecological significance of different visual pigments in the dorsal and ventral segments may be related to the bullfrog's tendency to be semi-submerged, exposing themselves to both aquatic and terrestrial environments simultaneously (see Reuter *et. al.*, 1971). The possible physiological significance of having a labile visual system is at present a topic for further investigations (for reviews, see Munz and McFarland, 1977; Knowles and Dartnall, 1977e).

The transition from porphyropsin rich tadpole retina to rhodopsin rich retinas in the adult frog has been cited as an example for the evolution of the visual system in the vertebrates (Wald, 1945-6; Crescitelli, 1972). The present discovery that bullfrog dorsal retinas may respond to light and temperature treatments with a change in the visual pigment composition, similar to paired visual pigment fishes and tadpoles whereas ventrals remain rhodopsin rich (similar to most other adult frogs) provides an additional system for studying the mechanism controlling the composition of visual pigments in the vertebrate retina.

Visual pigment and vitmain A composition in the eye

There have been relatively few studies comparing the visual pigment composition in the retinas and vitamin A composition in the pigment epithelium of animals. The results of Wald (1939, on chinook salmon, *Oncorhynchus tshawytscha;* rainbow trout, *Salmo gairdneri* and brook trout, *Salvelinus fontinalis*), Wilt (1959, on bullfrog tadpoles) and Reuter *et. al.*, (1971, on bullfrog) suggest that the vitamin A composition of the bleached retina and the pigment epithelium are alike. These results are difficult to assess because the vitamin A composition of the bleached retina and the visual pigment composition of the retina may not necessarily be the same (see General Introduction). Bridges and Yoshikami (1970b) observed that the proportions of rhodopsin and porphyropsin in rudd retinas were associated with similar proportions of retinol and 3-dehydroretinol in the pigment epithelium. Direct and quantitative comparisons of visual pigments (in the retina) and vitamin A (in the pigment epithelium) later were reported for amphibians (Bridges, 1975) and for goldfish (Chapter III). By plotting percent 3-dehydroretinol in the pigment epithelium against percent porphyropsin in the retina, the vitamin A composition may only 'match' the visual pigment composition when the tadpoles (bullfrog and bronze frog) and adult (bullfrog) were kept under light (see Fig. 12, Bridges, 1975). In constant darkness, 3-dehydroretinol proportions in the pigment epithelium were significantly higher than the porphyropsin proportions in the retinas of tadpoles (Bridges, 1975). Moreover, 3-dehydroretinol proportions in the pigment epithelium of the goldfish, kept under light/dark conditions, were always high (exceeding 85% 3-dehydroretinol) regardless of the visual pigment composition (ranging from less than

10% porphyropsin to more than 90% porphyropsin) in the retina. The results of the present study on bullfrog ocular tissues agree with Bridges' (1975) conclusion that visual pigment composition in the retina does not always match the vitamin A composition in the pigment epithelium.

One important difference between the experiments of Bridges' (1975) and the present study and those of Wald (1939) and Reuter *et. al.*, (1971) is that the former induced visual pigment changes by manipulating light (and temperature) whereas the latter employed animals from natural populations. Is it possible, that light and temperature can induce a change in visual pigment ratio in the retina that is not dependent on a concomitant change in vitamin A composition of the pigment epithelium? My earlier experiment on the goldfish visual system (Chapter III) has confirmed this suggestion (see Fig. 7, Chapter III). It shows that the degree of 'resemblance' between the percentage of porphyropsin and the percentage of 3-dehydroretinol is dependent on the length of acclimation of the animal to a particular light and temperature environment. It is likely that animals from natural populations (Wald, 1939; Reuter *et. al.*, 1971) alter their visual pigment and vitamin A composition at a different rate than those subjected to artificial (and abrupt changes in) light/temperature environments (Bridges, 1975; present study) resulting in discrepancies in findings. Whether the retina can selectively sequester one of the two vitamins A from the pigment epithelium for pigment synthesis or can procure its vitamin A from a location other than the pigment epithelium is not known at present (see Bridges, 1975). Alternatively, since my method measured both the free and esterified vitamins A in

the pigment epithelium together, there may be a small pool of free vitamin A_1 and A_2 whose proportions were similar to those of the rhodopsin and porphyropsin in the retina. The visual pigment composition would then be dependent on the free vitamin A_1 and A_2 ratio, which could be quite unrelated to the total vitamin A_1 to A_2 ratio in the pigment epithelium. The answer to these questions awaits further studies.

That the percent 3-dehydroretinol in the pigment epithelium 'followed' the change in visual pigment composition in the retina suggests that vitamin A storage in the pigment epithelium reflects the events occurring in the retina. Since goldfish have an enormous storage of vitamin A in their epithelium (41 moles per mole of visual pigment, Bridges, 1975), it is not surprising that a lengthy period is required before the two tissues have similar proportions of vitamin A_1 and A_2. It would be of interest to see, however, whether a comparable time period is required to alter the vitamin A_2 proportion in the pigment epithelium of the bullfrogs which have relatively less storage vitamin A (2 moles per mole of visual pigment, Bridges, 1975). Although I did not perform this experiment, comparison of Fig. 10 of the study on the bullfrog and Fig. 6 of the study on the goldfish (Chapter III) may suggest an answer. After 40-50 days of acclimation, rhodopsin rich bullfrogs had 25.9-42.4% vitamin A_2 in their (dorsal) pigment epithelium (Fig. 10) whereas the rhodopsin rich goldfish had more than 85% 3-dehydroretinol in their pigment epithelium (Fig. 6, Chapter III). This is suggestive that the time period required to alter the vitamin A composition may be related to the differences in the amount of vitamin A stored in

the pigment epithelium in different species.

The current knowledge on the relationship between visual pigments in the retina and vitamin A in the pigment epithelium is inadequate to satisfactorily explain the mechanism of visual pigment conversion. It appears that by studying visual pigment changes, one may also gain insight into how the retina procures its vitamin A for visual pigment synthesis. This is important in the understanding of the biochemistry of the visual cycle in the vertebrates. The earlier suggestion that the formation of vitamin A_2 from A_1 'must' occur in the pigment epithelium via a '3-4 dehydrogenase' needs to be documented (Reuter *et. al.*, 1971; Bridges and Yoshikami, 1970b).

CHAPTER V

GENERAL DISCUSSIONS

Since the discovery of visual pigment changes in the rudd (Dartnall *et. al.*, 1961), researchers working on visual pigment conversions have encountered difficulty in the manipulation of rhodopsin/porphyropsin ratios in paired pigment species. This refers, for example, to the shifting of the visual pigment composition in fishes during the transfer from an outdoor to an indoor situation (Allen, 1971; Jacquest and Beatty, 1972). This lack of stability of the visual pigment composition often resulted in difficulties in the design of an experiment which required the comparison between a specific light, temperature or hormonal treatment and a control. For example, Allen (1971) collected golden shiners from Oregon coastal streams to study the effect of light quality (wavelength) on the visual pigment composition. Upon arrival, these shiners had 90.6% porphyropsin. After 15 days of treatments with lights of different wavelength (and total darkness), groups of shiners had mean percent porphyropsin ranging from 57.2 (constant dark) to 78.2 (blue light). However, shiners in the natural habitat also lowered their percent porphyropsin to 63.1 when they were re-sampled. In addition, a similar decrease of porphyropsin percentages was encountered in the 'control' group (to 62.9%, fish were held under indoor room light). This suggests that the effect of light treatment acted together with an unidentified 'effect', both causing the porphyropsin ratios to decrease in the shiners. Whether this unidentified 'effect' interacts with the effect of a specific treatment is difficult to resolve. Similar cases can also be

found in the literature (Jacquest and Beatty, 1972).

The present study shows that the visual pigment composition of rainbow trout will stablize when fish are subjected to constant light and temperature treatments for a 30-50 day period (Fig. 1, Chapter II). This steady-state visual pigment composition not only can serve as a reliable index for the effects of light and temperature on the visual pigment composition of trout (Fig. 2, Chapter II), but also can be used as a stable 'control' to which the effect of a particular treatment is compared. An appropriate example is given in Chapter III (Fig. 5). Upon arrival, goldfish (with high porphyropsin ratios) were placed in a condition favoring low porphyropsin until their visual pigment compositions were stablized. Thyroxine was then applied to a treatment group while the visual pigment composition of the control group remained unchanged. The effect of thyroxine was then confirmed when the visual pigment composition of the treatment and the control groups were compared (Fig. 5, Chapter III).

This ability to predictably manipulate visual pigment composition has other advantages. One example is the present discovery of rhodopsin in the goldfish. From earlier studies (Allen and McFarland, 1973; Cristy, 1974; Tsin, 1976; Tsin and Beatty, 1977a; McFarland and Allen, 1977), it was concluded that higher water temperature will favor rhodopsin irrespective of the species' response to light (see General Introduction). Therefore, by subjecting goldfish (of high porphyropsin ratios) to higher temperature, I was able to increase rhodopsin proportions in order to confirm its presence. Goldfish were subjected to a high water temperature (28^0C) at an arbitrarily chosen photoperiod (16L/8D)

and light intensity (under the illumination of a 60W light bulb) for a 30-36 day period. Partial bleaching experiments performed on the retinas from these light and temperature treated goldfish confirmed the presence of a large proportion of rhodopsin, thus allowing an accurate identification of this visual pigment (see Method of Chapter III).

Further experiments on the goldfish visual pigment system indicated that a certain light intensity (under the illumination of a 7.5W light bulb) and photoperiod (16L/8D), in addition to warm water (30^0C) conditions may be required for high rhodopsin proportions (Fig. 4a, b and c; Chapter III). This suggests that a preliminary survey of visual pigments in a species, suspected of exhibiting changes in visual pigment proportions, should also include regimes of different light intensity, photoperiod and temperature. This suggestion was put to a test in the study of the visual pigment system of the bullfrog.

Adult bullfrogs were thought to possess only rhodopsin (Wald, 1937-8; Crescitelli, 1958, 1972). However, Reuter *et. al.*, (1971) reported the presence of a porphyropsin, in addition to rhodopsin, in the dorsal retina of the adult bullfrog. Moreover, they also noted that the winter frog had higher proportions of porphyropsin in their retinas (about 40%) than the summer frog (about 5% porphyropsin). Therefore, it was of interest to see whether this discrepancy in the literature about bullfrog visual pigments (i.e. bullfrogs have only rhodopsin as suggested by Wald, 1937-8; Crescitelli, 1958, 1972 versus bullfrogs have a rhodopsin/porphyropsin pair as suggested by Reuter *et. al.*, 1971) is the result of light and temperature influences on the photopigment composition in the retina. From the results of the present study

(Chapter IV), it is possible to suggest that this disagreement in the literature on bullfrog visual pigments may be caused by light and temperature since these environmental factors significantly altered the visual pigment composition in bullfrogs used in the present study (Chapter IV). Consequently, the adult bullfrog may constitute another example, in addition to the goldfish, of an animal, earlier identified as a single pigment species (i.e. the goldfish was thought to possess only porphyropsin, Schwanzara, 1967; Bridges, 1973; the adult bullfrog was reported to have only rhodopsin, Wald, 1937-8; Crescitelli, 1958, 1972), that is now confirmed to possess paired and labile visual pigments (see Chapter III for the goldfish and Reuter *et. al.*, 1971 and Chapter IV for the bullfrog).

The temporal change of visual pigment proportions in trout indicated (Chapter II) that almost equal amounts of time are required to replace porphyropsin by rhodopsin in the dark and to replace rhodopsin by porphyropsin under continuous light. This suggests that the bleaching and resynthesis cycle under illumination did not significantly elevate the rate of visual pigment conversion. This infers that visual pigment turnover may be regulated by a change in the proportion of new visual pigment synthesized at the base of the rod outer segment, a process that occurs in light and in darkness (Young, 1967; Besharse, Hollyfield and Rayborn, 1977). This suggestion is substantiated by the agreement of the time required to completely replace discs of the rod outer segment in the frog (*Rana pipiens*, 6-7 weeks, Young, 1967) and the time required to replace rhodopsin by porphyropsin (or vice versa) in trout (30-50 days, Fig. 1, Chapter II). Moreover, Loew and Dartnall (1976), using microspectrophotometry, have concluded that rudd subjected

to continuous darkness (a condition favoring rhodopsin in the rudd) were richer in rhodopsin at the base of the rod outer segment than at its apical end.

Bridges and Yoshikami (1970b) suggested that the relative proportion of visual pigment synthesized at the base of the rod outer segment may be related to the relative availability of the two vitamins A in the pigment epithelium. This suggestion was primarily based on the finding that the visual pigment composition in the retina and the vitamin A composition in the pigment epithelium are similar in the rudd (Bridges, and Yoshikami, 1970b). The present study suggests that this view should be modified because the visual pigment composition in the retina of the goldfish does not match the vitamin A composition in the pigment epithelium (Fig. 6, Chapter III). Moreover, further studies showed that the length of acclimation of an animal (Chapter III, Fig. 7) and the amount of storage vitamin A in the pigment epithelium (see Discussion of Chapter IV) may play an important role in the degree of resemblance of vitamin A composition in the pigment epithelium and visual pigment composition in the retina.

The present comparative study on the visual pigments and vitamins A in fishes and amphibians has demonstrated the apparent inadequacy of our understanding on the vertebrate visual system. Further studies are needed to relate this laboratory finding on visual pigment conversion to the ecological significance of animals in the natural habitat. It may also be of interest to study whether any other species, reported to possess only rhodopsin or porphyropsin, also possess paired visual pigments. The mechanism of visual pigment conversion may possibly be elucidated via an extensive study on the hypothesized enzyme

'3-dehydrogenase' which presumably, interconverts the two vitamins A.

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APPENDIX 1

Visual Pigment Change in Three Groups of Rainbow Trout (Groups A, B and C) in Relation to Time after Transfer to 24L(6^0C) and 24D(18^0C)

Porphyropsin percentages in the retinas and 3-dehydroretinol percentages in the (pooled) livers at different sampling dates, are indicated in the table. Means± 1 S.E. (sample size) are indicated in the table. For details, see Methods in Chapter II. The initial porphyropsin percentages for groups A,B and C (see day 0 in the table) were obtained after preconditioning trout to 24L(6^0C), 12L/12D (12^0C) and 24D(18^0C) for 45 days.

	Number of Days						
	0	10	20	30	40	60	80
Group A in 24L							
% porphyropsin	83.4±2.9(5)	92.8±1.7(4)	86.4±4.3(5)	89.1±1.8(5)	89.9±1.9(5)	91.7±1.8(5)	91.8±1.3(6)
% 3-dehydroretinol	85.1	82.4	86.8	88.0	84.6	77.9	86.0
Group A in 24D							
% porphyropsin	83.4±2.9(5)	52.9±2.7(5)	31.8±1.8(5)	31.4±4.9(4)	28.9±6.5(5)	18.5±1.7(4)	
% 3-dehydroretinol	85.1	91.3	83.4	92.9	86.1	82.9	
Group B in 24L							
% porphyropsin	67.8±5.7(5)	74.9±4.4(5)	84.3±4.6(5)	86.7±2.4(5)	90.2±3.3(5)	90.3±3.7(5)	95.8±0.6(7)
% 3-dehydroretinol	84.1	63.8	81.2	83.1	85.5	75.9	77.4
Group B in 24D							
% porphyropsin	67.8±5.7(5)	44.0±3.3(5)	35.1±5.7(5)	25.8±10 (5)	14.5±5.8(5)	12.9±1.9(9)	
% 3-dehydroretinol	84.1	76.6	88.4	91.3	77.5	78.3	
Group C in 24L							
% porphyropsin	16.5±3.3(5)	59.4±4.8(5)	79.0±5.4(5)	91.7±2.6(5)	87.1±2.7(5)	92.9±1.9(5)	91.3±4.5(3)
% 3-dehydroretinol	78.2	87.8	87.0	85.3	79.7	77.0	
Group C in 24D							
% porphyropsin	16.5±3.5(5)	14.3±5.6(5)	15.7±6.7(5)	16.2±5.7(5)	12.9±3.8(4)		
% 3-dehydroretinol	78.2	89.2	90.1	85.4	89.8		

APPENDIX 2

Steady-State Visual Pigment Composition of Rainbow Trout

Porphyropsin percentages and total visual pigment (in absorbance units in extracts of equal volume) in the retinas and 3-dehydroretinol percentages in the liver were examined after trout had been acclimated for 60 days in different light-temperature regimes. Some vitamin A decomposition was experienced in the liver stored (-20°C) for several months before analysis. Means± 1SE (sample size) are indicated in the table. For details, see Methods in Chapter II.

		% porphyropsin	Absorbance	% 3-dehydroretinol (liver)
24L	6°C	†91.3±1.4(15)	.12±.01(15)	
	12°C	74.2±2.3(18)	.16±.01(18)	60.7±3.5(6)
	18°C	61.2±2.3(20)	.18±.01(20)	76.0±4.5(9)
18L	6°C	*80.3±2.3(12)	.20±.01(12)	
	12°C	*60.2±3.2 (9)	.23±.02 (9)	
	18°C	*49.3±2.7(10)	.23±.01(10)	
12L	6°C	78.3±2.6(20)	.15±.01(20)	69.0±4.6(6)
	12°C	53.5±2.0(18)	.17±.01(18)	57.9 (2)
	18°C	45.9±3.1(18)	.19±.01(18)	
6L	6°C	71.8±2.9(20)	.15±.01(20)	
	12°C	39.4±3.9(11)	.18±.01(11)	56.3 (3)
	18°C	*30.8±4.0(19)	.20±.01(19)	
24D	6°C	38.2±3.4(29)	.17±.01(29)	
	12°C	30.5±3.5(30)	.23±.01(30)	56.9 (1)
	18°C	13.0±1.8(29)	.25±.01(29)	63.2 (1)
	18°C	†14.6±2.2(13)	.26±.02(13)	

* These results were obtained in the winter 1978/79, instead of in the summer of 1977, as were for the rest.
† These are means from appropriate values (60th day) in Appendix 1.

APPENDIX 3

Visual Pigment and Vitamin A Composition in Goldfish Subjected to Different Light-Temperature Regimes for 50 Days

Porphyropsin percentages, total visual pigment (in absorbance units in extracts of equal volume), 3-dehydroretinol percentages in the pigment epithelium and in the pooled livers are presented. Means± 1SE of 13 fish per group are indicated in the table. For details, see Methods in Chapter III.

					% 3-dehydroretinol	
			% porphyropsin	Absorbance	Pigment Epithelium	Pooled Livers
24L	7.5W	30°C	90.5±1.1	.27±.02	89.4±1.1	77.4
16L/8D	7.5W	30°C	9.8±1.7	.29±.02	80.9±1.1	68.8
6L/18D	7.5W	30°C	84.6±1.2	.32±.02	92.8±1.5	76.0
24D		30°C	88.5±1.1	.29±.02	90.6±1.3	81.2
16L/8D	dimmed 7.5W	30°C	23.8±3.4	.35±.02	82.3±2.2	74.3
16L/8D	2X15W	30°C	36.3±3.4	.27±.02	82.9±1.6	78.2
16L/8D	2X60W	30°C	57.7±2.5	.24±.01	83.4±1.2	73.8
16L/8D	7.5W	20°C	89.4±0.8	.21±.02	90.7±1.1	75.3
16L/8D	7.5W	10°C	91.5±0.8	.12±.01	90.3±1.8	58.5

APPENDIX 4

Effect of Thyroxine on the Visual Pigment and Vitamin A Composition of the Goldfish

Porphyropsin percentages, total visual pigments (in absorbance units in extracts of equal volume), 3-dehydroretinol percentages in the pigment epithelium and in pooled livers are presented. Means± 1SE (sample size) are indicated in the table. These goldfish were pre-conditioned at 16L/8D by a 7.5W light bulb in 28°C water for 29 days before the thyroxine (L-thyroxine introduced into the tank water at a concentration of 100µg/liter tank water) was introduced on the 30th day (i.e. Day Zero in the table). For details, see Methods in Chapter III.

	% porphyropsin	Absorbance	% 3-dehydroretinol	
			Pigment Epithelium	Pooled Livers
Day Zero	38.0±5.4 (8)	.24±.01 (8)		79.2
Day 10th				
Control	27.8±5.3 (8)	.32±.02 (8)	83.7±2.0 (8)	58.1
Thyroxine Treated	89.2±2.0 (8)	.24±.03 (8)	90.3±1.8 (8)	76.4
Day 20th				
Control	28.7±2.6 (8)	.29±.02 (8)	83.3±2.3 (8)	78.6
Thyroxine Treated	90.0±1.5(12)	.22±.02(12)	91.6±1.0(12)	78.3

APPENDIX 5

Change of Visual Pigment and Vitamin A Composition with Time in the Goldfish

Porphyropsin percentages, total visual pigments (in absorbance units in extracts of equal volume), 3-dehydroretinol percentages in the pigment epithelium and in pooled livers are presented. Means± 1SE (sample size) are indicated in the table. These goldfish were held in a holding tank (250 liters) under a 15W light bulb at 16L/8D in 28°C during the experiment. For details, see Methods, Chapter III.

	% porphyropsin	Absorbance	% 3-dehydroretinol	
			Pigment Epithelium	Pooled Livers
Day Zero	93.3±0.5(10)	.14±.01(10)	88.7±3.3(10)	
Day 50	28.7±2.6 (8)	.29±.02 (8)	83.3±2.3 (8)	78.6
Day 150	23.8±3.7 (7)	.30±.02 (7)	68.9±1.1 (7)	51.9
Day 300	18.0±1.9 (8)	.28±.02 (8)	49.9±2.6 (8)	53.5

APPENDIX 6

Visual Pigment and Vitamin A Composition in Leopard Frogs Subjected to Different Light-Temperature Regimes for 30 Days

Porphyropsin percentages, total visual pigments (in absorbance units in extracts of equal volume) and 3-dehydroretinol percentages in the pooled livers are presented. Mean and sample size are indicated in the table. (+) sign denoted small amount of 3-dehydroretinol existed (less than 10%) in the samples. For details, see Methods in Chapter IV.

		% porphyropsin	Absorbance	% 3-dehydroretinol in pooled livers
6°C	6L/18D 7.5W	0(3)	.17(3)	+
	2X60W	0(2)	.21(2)	+
	18L/6D 7.5W	0(3)	.31(3)	+
	2X60W	0(1)	.29(1)	+
30°C	6L/18D 7.5W	0(3)	.24(3)	+
	2X60W	0(3)	.31(3)	+
	18L/6D 7.5W	0(3)	.39(3)	+
	2X60W	0(4)	.37(4)	+

APPENDIX 7

Visual Pigment and Vitamin A Composition in the Bullfrog Kept Under Different Light-Temperature Regimes for 40 Days

Porphyropsin percentages of dorsal (D) and ventral (V) and whole (W) retinas for individual frogs are presented. Percent porphyropsin value of dorsal (or ventral) retina was obtained by averaging the results from determinations of left dorsal (or ventral) and right dorsal (or ventral) retinas of an individual. Value for the whole retinas was then calculated from the weighed sum of the dorsal and ventral retinas of an individual, i.e. taking into consideration the relative contribution of visual pigment amounts from the dorsal and ventral segments. Also in the table are total visual pigment amount (in absorbance units), vitamin A_2 percentages in the pigment epithelium and 3-dehydroretinol percentages in the liver from each individual. (+) sign denotes small amount of 3-dehydroretinol (less than 10%). For details, see Methods in Chapter IV.

	Individual		% porphyropsin	Absorbance	% vitamin A_2 Pigment Epithelium	% 3-dehydroretinol Liver
18L/6D	1	D	51.1	.15		
7.5W		V	12.7	.20		+
10°C		W	28.8	.35		
	2	D	52.5	.15		
		V	13.7	.18		+
		W	31.8	.33		
18L/6D	1	D	3.0	.07		
7.5W		V	1.9	.17	39.0	+
30°C		W	2.2	.24		
	2	D	6.0	.19	33.2	
		V	3.8	.27	21.2	+
		W	4.7	.46		
18L/6D	1	D	86.1	.12	100.0	
2X60W		V	16.8	.14		+
10°C		W	48.0	.26		
	2	D	78.4	.12	96.7	
		V	27.4	.13		+
		W	51.6	.25		
18L/6D	1	D	6.0	.15	35.3	
2X60W		V	1.9	.14	40.1	+
30°C		W	4.0	.28		
	2	D	7.3	.18		+
		V	4.9	.34	22.9	
		W	5.7	.53		
6L/18D	1	D	40.4	.16	48.2	
7.5W		V	6.2	.20	13.6	+
10°C		W	18.7	.35		
	2	D	36.4	.17		+
		V	6.2	.15	54.1	
		W	20.2	.31		
6L/18D	1	D	3.8	.24	25.9	
7.5W		V	3.0	.30	7.0	+
30°C		W	3.7	.54		
	2	D	7.3	.12	42.3	
		V	0.0	.16	6.0	+
		W	6.1	.28		
6L/18D	1	D	72.9	.13	78.8	
2X60W		V	12.0	.20		+
10°C		W	35.8	.33		
	2	D	69.9	.10	59.0	
		V	8.5	.14	12.5	+
		W	34.2	.24		

APPENDIX 7 (continued)

	Individual		% porphyropsin	Absorbance	% vitamin A_2 Pigment Epithelium	%3-dehydroretinol Liver
6L/18D	1	D	6.2	.20		
2X60W		V	4.9	.34	0.0	+
30°C		W	5.4	.54		
	2	D	7.3	.09		
		V	3.8	.35		+
		W	4.5	.43		
18L/6D	1	D	80.2	.15		
2X60W		V	11.1	.23		+
20°C		W	38.6	.38		
	2	D	78.4	.14	91.6	
		V	15.0	.25		+
		W	37.5	.39		
24L	1	D	91.6	.13	74.6	
2X60W		V	30.2	.33	10.9	+
10°C		W	48.4	.46		
	2	D	77.2	.14	72.7	
		V	17.9	.26		+
		W	39.0	.41		
12L/12D	1	D	87.5	.13	78.4	
2X60W		V	23.2	.27	41.3	+
10°C		W	44.6	.40		
	2	D	79.3	.10	79.9	
		V	17.9	.15		+
		W	41.8	.25		
24D	1	D	53.0	.14	69.8	
10°C		V	20.1	.30	48.8	+
		W	30.6	.44		
	2	D	40.7	.13	87.1	
		V	8.2	.19		+
		W	21.3	.32		

APPENDIX 8

Light Qualities of Fluorescent Tubes and Tungsten Bulbs

In this thesis, both fluorescent tubes (Chapter II) and tungsten bulbs (Chapter III and IV) were employed. The spectral energy (in $\mu W/cm^2$-sec/nm) were measured at water surface (between 400-750nm), using the remote probe of a spectroradiometer (ISCO, model SR, Instrumentation Specialties Co., Nebraska, 68505). These readings were converted to spectral irradiance (in photons/cm^2-sec/nm) using the equation of Planck's (Wyszecki and Stiles, 1967):

$$n = \frac{I \times \lambda}{h \times c}$$

where n = spectral irradiance

I = spectral energy

λ = wavelength

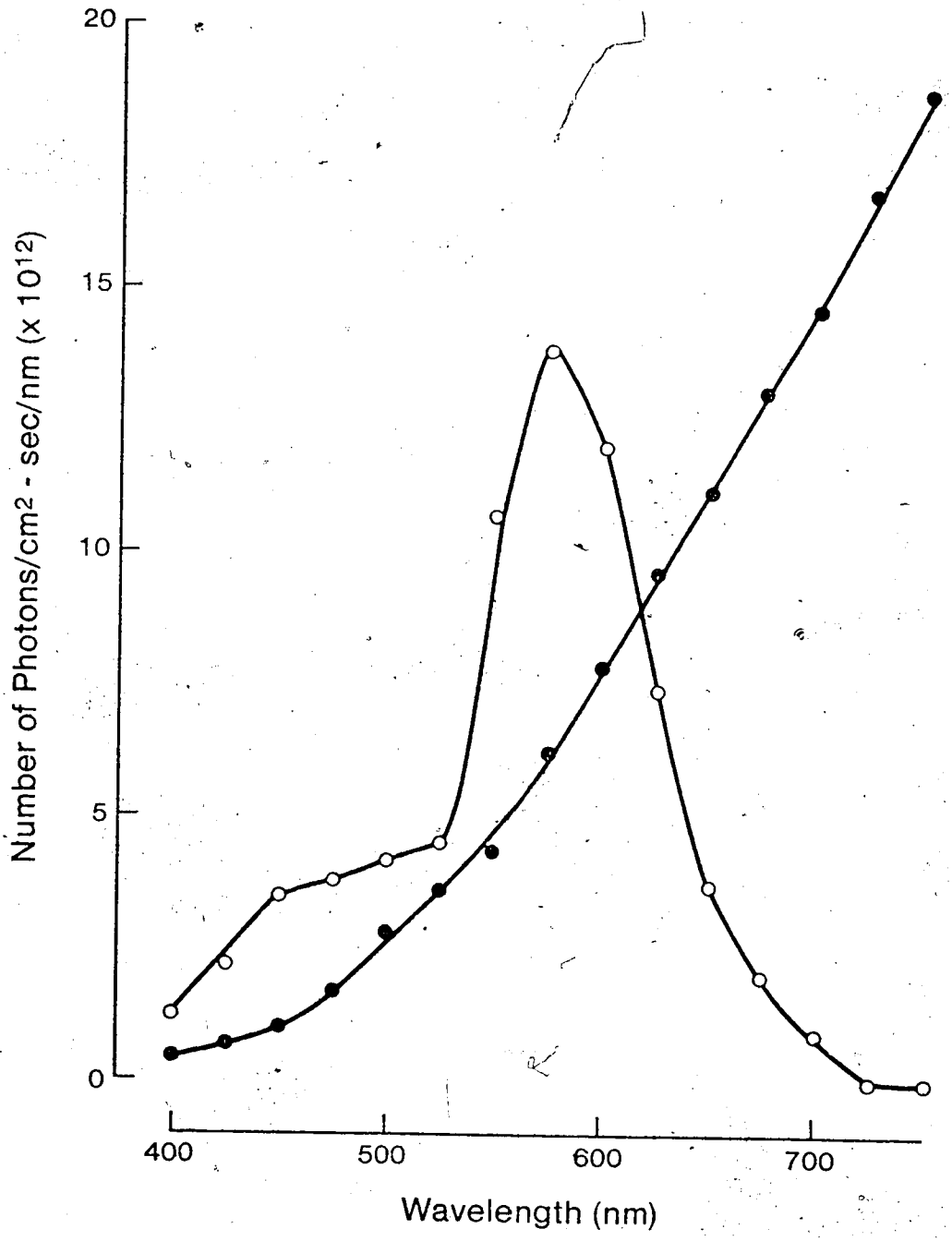
h = Planck's constant

c = velocity of light

The spectral irradiance (photons/cm^2-sec/nm) was then plotted against the wavelength (nm) and the area under the curve represents the total irradiance (i.e. total number of photons/cm^2-sec, between 400-750nm). Figure 11 shows an example of this.

Fig. 11 Spectral irradiance delivered by fluorescence tubes and tungsten bulbs.

Appendix 8 describes how the spectral irradiance of the two light sources were derived. Total irradiance (between 400-750 nm) were obtained by integrating the area beneath the curve or by paper weighing. Using the latter method, the total irradiance by the two 20W fluorescent tubes (open circles) was 1.77×10^{15} photons/cm^2-sec. (between 400-750 nm) whereas the two 60W tungsten bulbs (filled circles) gave a total irradiance of 2.44×10^{15} photons/cm^2-sec. (between 400-750 nm).


20
15
10
5
0
Number of Photons/cm2 - sec/nm (x 1012)
400
500
600
700
Wavelength (nm)