Department of Biosciences
Division of Physiology and Neuroscience
University of Helsinki
and
Department of Biomedical Engineering and Computational Science
Aalto University School of Science
Finland

FUNCTIONAL PROPERTIES OF VISUAL PIGMENTS USING A1 AND A2 CHROMOPHORE: FROM MOLECULES TO ECOLOGY

Pia Saarinen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, for public examination in lecture room 2041, Biocenter 2, Viikinkaari 5, on 17 August 2012, at 12 o'clock.

Helsinki 2012

Supervised by:
Prof. Kristian Donner, PhD
Department of Biosciences
Division of Physiology and Neuroscience
University of Helsinki
Finland

and
Prof. Ari Koskelainen, Dr. Sci. Techn.
Dept. of Biomedical Engineering and Computational Science
Aalto University
Finland

Reviewed by:
Prof. Eric Warrant, PhD
Biologiska institutionen
Lund University
Sweden

and
Prof. Vladimir Kefalov, PhD
Department of Ophthalmology
Washington University in St. Louis School of Medicine
USA

Opponent:
Prof. Justin Marshall, PhD
Sensory Neurobiology Group
Queensland Brain Institute
University of Queensland
Australia

Custos:
Prof. Juha Voipio, PhD
Department of Biosciences
Division of Physiology and Neuroscience
University of Helsinki
Finland

ISBN 978-952-10-8130-9 (pbk.)
ISBN 978-952-10-8131-6 (PDF)

Unigrafia
Helsinki 2012

To my children,

ACKNOWLEDGEMENTS

I'm extremely grateful to my supervisor Professor Kristian Donner for making this thesis possible. For the whole decade which it took, he never stopped believing in it although I occasionally had my doubts. His guidance and incredible expertise together with the enjoyable discussions and his emphatic and humoristic attitude have meant a great deal to me. I'm also grateful to my other supervisor Professor Ari Koskelainen who has very kindly explained to me the mysteries of physics, and has always kept his lab door open for me. He and his team has been a true motivator through these years.

I want to thank Professor Juha Voipio for all his support and the advice that he has given me. He has been an excellent superior; fair, easy to approach, and he has always been very positive towards me.

I'm very grateful to my lab friends whom I've spent so much time with. Nanna, Lotta and Chrisse introduced me to vision science. Then came Mirka, who has become a real friend! Together we have solved a number of technical and theoretical research problems, not to mention the "other kind" of problems, too. Juissi has been very sympathetic, excellent company and he has been a true supporter. Henry, Antti, and Sirpa have had a positive impact on my work and my life in general. There have also been many others, like Martta, Cecilia and Erica, whose company I have found spiriting. Thank you all!

I want to thank all my friends in Otaniemi. Petri (presently in Viikki) is someone who is so oriented to science that I have never met anyone else like him. It's simply impossible not to be positively infected from his motivation and spirit. Working with Rauli has been fun and I always enjoy science at least twice as much when he is around. With Hanna, we have had many fruitful discussions in the ERG lab, in ARVO, and in many other places. She has given me many new ways to look at both science and life. Frans has also been very kind and helpful towards me both at work and during free time. Soile has shared her skills in science and her confidence towards life with me. It has also been a joy to watch the talented younger scientists Teemu and Marja.

During all these years first in Elukka and later in Viikki, I have become acquainted with many important people. All the coffee breaks with Riitta have often been more than necessary to cope with my work and life! I have had numerous enjoyable moments with Sissi, Segge, Anou, Jossu, Sinikka, Klaus, Eva, Reijo, Annika and all the others.

Especially big thanks are due to Dr. Magnus Lindström for the wonderful times in Tvärminne Biological Station, both during Visionarium seminars and during my stay there as I was writing this thesis. I want to thank Dr. Victor Govardovskii for his advice and help with MSP. Prof. Em. Tom Reuter

I want to thank for good discussions and support. And I'm very grateful to my pre-examiners, Prof. Vladimir Kefalov and Prof. Eric Warrant who in a very tight schedule reviewed this thesis.

Lastly, I want to sincerely thank my family. Jarmo and Jyrki with his family have always supported and encouraged me. My mother has given me the basic grounds and attitude towards work and life. Without her belief in me, I couldn't have done this work. My dear husband Teemu has been very patient with my work. He has been proud of my achievements and he has always loved me without reservation. He has been the solid rock of my life. But the most important achievements in my life are my children Vera, Sara, and Heidi. The power that they give me every day is the most important thing of all! I love you so much!

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to by Roman numerals (I-III), and on unpublished results:

I Ala-Laurila P., Saarinen P., Albert R., Koskelainen A. and Donner K. (2002). Temperature effects on spectral properties of red and green rods in toad retina. Vis. Neurosci. 19:781–792.

II Ala-Laurila P., Albert R., Saarinen P., Koskelainen A. and Donner K. (2003). The thermal contribution to photoactivation in A2 visual pigments studied by temperature effects on spectral properties. Vis. Neurosci. 20:411–419.

III Saarinen P.*, Pahlberg J.*, Herczeg G., Viljanen M., Karjalainen M., Shikano T., Merilä J., and Donner K. (2012). Spectral tuning by selective chromophore uptake in rods and cones of eight populations of nine-spined sticklebacks (*Pungitius pungitius*). J. Exp. Biol. (in press)

*These authors contributed equally to the work.

The author's contribution to the studies included in this thesis:

I The author did most of the MSP recordings and the MSP analysis and participated in the ERG experiments and in writing the manuscript. The article has been included in the Dr.Sci.Techn. Thesis of Petri Ala-Laurila (PAL), presented at Helsinki University of Technology. PAL did most of the ERG recordings and ERG analysis and participated in writing the manuscript.

II The author participated in the MSP recordings and analysis and some of the ERG recordings. The article has been included in the thesis of PAL (see above), who participated in the ERG and MSP recordings, did most of the ERG analysis, and had the main responsibility for writing the manuscript.

III The author participated in developing the idea of the study and had the main responsibility for writing the manuscript. She did all the MSP recordings of 2011 and all the MSP analysis, as well as the recordings and analysis of water transmission spectra in several stickleback habitats in different parts of Finland.

ABBREVIATIONS

A1	retinal (as chromophore in a visual pigment)
A2	3,4-dehydroretinal (as chromophore in a visual pigment)
ERG	electroretinogram
G_t	visual G protein (transducin)
IR	infrared
IRBP	interreceptor retinoid binding protein
λ_{max}	wavelength of maximum absorbance
L-cone	long-wavelength sensitive cone
LWS	long-wavelength sensitive cone type opsin
M-cone	middle-wavelength sensitive cone
MSP	microspectrophotometry
MWS	middle-wavelength sensitive type opsin
N_B	background photoactivations
N_S	signal photoactivations
OD	optical density
PCR	polymerase chain reaction
QC	quantum catch
Rh	rhodopsin
Rh*	activated rhodopsin
RH1	opsin of vertebrate rods
RH2	rhodopsin 1 like opsin
RPE	retinal pigment epithelium
S-cone	short-wavelegth sensitive cone
SD	standard deviation
SEM	standard error of the mean
7-TM	seven transmembrane (receptor)
SNR	signal-to-noise ratio
SWS1	short-wavelength sensitive type 1 opsin
SWS2	short-wavelength sensitive type 2 opsin
UV	ultraviolet

ABSTRACT

The relation between two fundamental functional variables of visual pigments, spectral absorbance and activation energy, was studied in 7 species of photoreceptors with pigments that differ in chromophore use (A1 or A2) and/or absorbance spectra. Spectra were characterized by the wavelength of peak absorbance (λ_{max}) determined by microspectrophotometry. Activation energies (E_a) were calculated from temperature effects on electrophysiologically measured spectral sensitivities at very long wavelengths. The A1 photoreceptors studied were the rhodopsin rods of the amphibians *Bufo bufo* (λ_{max} = 503 nm), *B. marinus* (504 nm) and *Rana catesbeiana* (502 nm) plus the "green" rod of *B. marinus* (433 nm); the A2 photoreceptors were porphyropsin rods of *R. catesbeiana* (525 nm) and crucian carp *Carassius carassius* (526 nm) plus the L-cones of *C. carassius* (619 nm).

There was a general inverse correlation between λ_{max} and E_a (r^2 = 0.84). Yet, in the three spectrally similar A1 rod pigments, estimated E_a varied from 44.3 ± 0.6 kcal/mol (*B. marinus*) through 46.5 ± 0.8 kcal/mol (*R. catesbeiana*) to 48.8 ± 0.5 kcal/mol (*B. bufo*). The use of A2 chromophore was consistently associated with lower E_a, the estimates being 44.2 ± 0.9 (*R. catesbeiana*), 42.3 ± 0.6 (*C. carassius* rods), and 38.3 ± 0.4 kcal/mol (*C. carassius* L-cones). At least in the case of the E_a difference between the two A1 and A2 pigments with the same opsin (*R. catesbeiana* rods, $P < 0.05$), the chromophore difference must be the actual cause. The lower activation energy associated with the A2 chromophore correlates with a higher level of thermal noise, liable to decrease visual sensitivity, as previously shown in the same A1-A2 pigment pair studied here (*R. catesbeiana* rods: Donner *et al.,* 1990) and more recently shown to be a general rule (Ala-Laurila *et al*. 2004, 2007; Luo *et al*. 2011).

Against this background, within-species adaptation of visual pigments to different light environments was studied in rods and cones of eight Fennoscandian populations of nine-spined stickleback (*Pungitius pungitius*), most of which have been isolated from each other since the end of the last glaciation (ca. 8000 years). Like many fishes, this species possesses a plastic A1-A2 chromophore system. The conceptual amino acid sequence of the rod opsin as derived from genomic DNA was identical in all populations, and the absorbance spectra of the four cone pigments suggested no differences in the cone opsins. However, there were significant differences in chromophore use between populations as studied in fish that had been kept in standardized aquarium conditions. Rods were A2-dominated in half of the populations and A1-dominated in the other half. In the A1-dominated group, the M-cones had significantly higher proportions of A2 than the rods, indicating differential chromophore use in rods and cones. Further, different

chromophore use of M- and L-cones was demonstrated in one population. The results show that varying chromophore proportions may be used for differential tuning of different photoreceptor types, arguably for high achromatic contrast sensitivity in dim-light, rod vision and good wavelength discrimination in daylight, cone vision. The unlikely ideal (a daunting task) would be if the proportions of more red-sensitive, noisy A2 and more blue-sensitive, stable A1 could be independently optimized in different photoreceptor types, observing different demands on spectral sensitivity and noise control.

1 INTRODUCTION

1.1 LIGHT AND VISION

1.1.1 LIGHT DISTRIBUTION ON LAND AND IN WATER

Light is a narrow band of the electromagnetic radiation spectrum with photon energies suitable for interaction with special receptor molecules, visual pigments, of animals. Potentially, it is the richest source of information for animals about their environment. In the daytime, a rain of solar photons, 6×10^{15} photons/s/mm^2, impinges on the top layer of Earth's atmosphere. This means that each square meter receives an effect equivalent to that from fourteen 100 Watt light bulbs. A considerable proportion of this radiation is reflected back into outer space or absorbed or scattered in all directions by clouds, atmospheric gases, vapours, and dust particles. At sea level, 14 % of the effect is lost during passage through a dry, clean atmosphere, and about 40 % in a moist or dusty atmosphere, compared to the value above the atmosphere (Moon 1961). The amount reaching the Earth's surface varies with the radiation wavelength: besides the so-called visible light, there is a relatively small amount of ultraviolet (UV), and a wide band of infrared (IR). Of this radiation, animal eyes are restricted to sensing only a narrow band from approximately 350 nm to 780 nm.

In addition to the effects of the air, the extent and type of cloud covers are of great importance in determining the amount of solar flux that penetrates to the Earth's surface. Even a thin layer of clouds, which does not obscure the sun, will decrease total irradiance (i.e., the radiant flux per unit area of a surface) by 5–10 %. A continuous thick sheet of clouds may transmit only 10 % of the solar radiation, about 70 % being reflected back into space and 20 % being absorbed. The difference between night and day is enormous. On a full moon, the night sky is about six log units less bright than in full sunlight, and the light is relatively richer in longer wavelengths. A starry sky with no moonlight is about 3 log units less bright than full moonlight, and even more red-shifted (Munz and McFarland 1973).

On land the differences in luminance between open habitats and the sky are modest. But in aquatic environments, the light is strongly attenuated in a wavelength dependent manner, decreasing exponentially with increasing depth. The loss in intensity is largely due to scattering, which is often more important than absorption in water. In pure and clear water (regardless of salinity), blue light ($\approx$ 475 nm) is transmitted the best (Clarke and James 1939). Water absorbs light more strongly at both shorter and longer wavelengths, but in the whole range from 300 nm to 500 nm the reduction in intensity due to absorbance in pure water does not exceed 5 % per metre of

water. For example, UV(A) radiation will penetrate clear water to a depth of several metres before it is appreciably diminished in intensity. For longer wavelengths reduction may be as high as 40% per metre, absorption growing until at least 750 nm (Pegau *et al.* 1997). The photon flux drops and the spectral band narrows with increasing depth, until approximately at 1000 meter depth, the sunlight has decreased below the limit where it can be used for vision by any animals.

The deep-blue colour of the open seas owes its blueness largely to selective Rayleigh scatter (causing an increased proportion of short wavelengths in upwelling light compared to downwelling light at the same depths) (Jerlov 1976). However, the picture differs in water bodies that contain various amounts of suspended or dissolved inorganic and organic matter (freshwater bodies, coastal sea waters). Especially, chlorophyll can dominate the colour of natural water, resulting in maximum transmission around 560-575 nm (Morel and Smith 1974). In this region of the spectrum chlorophyll has minimal absorption, but on the other hand the absorption of the water itself begins to increase rapidly. Another factor influencing the colour of the water are the yellow-brown humic substances dissolved in the water. They absorb especially short-wavelength light making many lakes look brownish compared with open seas. Together, absorption due to organic yellow substances (Gelbstoff) and water leaves mid-spectrum “green” light least affected. When yellow substance is present in high concentration, the water transmits most strongly at even longer wavelengths. Many variables affect the colour of the water: time of the year, water temperature (effect of productivity), and the quantities of nutrients, i.e., trophic state of the water (eutrophic/dystrophic lakes) (Loew and Lythgoe 1978). The effect of the above mentioned factors can limit penetration of sunlight to 30-50 metres in coastal waters, and to only a few meters in fresh waters (Bowmaker 1995).

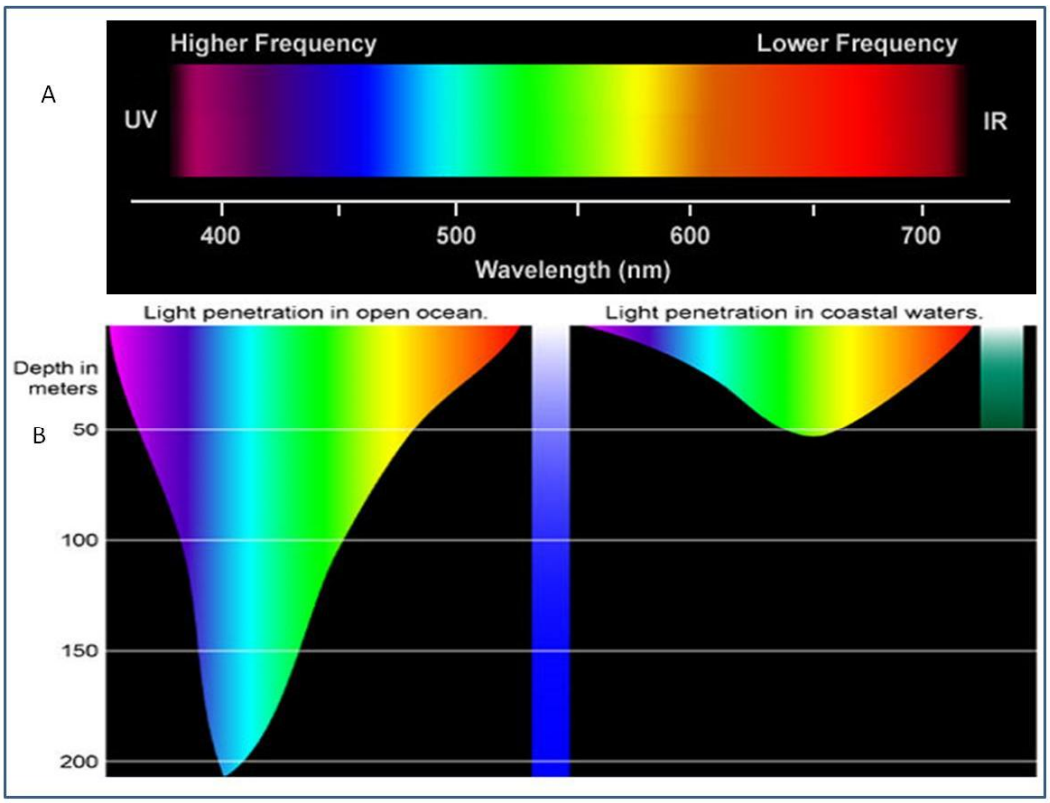


Fig. 1A At the blue end of the visible spectrum, the wavelength of light is shorter (about 400 nm). At the red end of the spectrum, the wavelength of light is longer (about 700 nm). Photon energy is proportional to frequency and thus inversely proportional to wavelength. (Image from http://photo.net/photo/edscott/vis00010.htm) **B** Light penetration in ocean and coastal water. (Image modified from http://oceanexplorer.noaa.gov/explorations/04deepscope/background/deeplight/media/diagram3.html)

1.1.2 VISION AND THE PHOTIC ENVIRONMENT

As evident from above, animal vision works in environments that vary enormously with respect to both the quantity and qualities of the available light. On the surface of the earth, the total photon flux varies by 10^{11}-fold from the brightest sunlight to a moonless, overcast night (Lythgoe 1979). In aquatic habitats, vision can work over 2-3 additional log units of intensity decreasing with increasing depth, before the abyssal regions, where the vision that remains in some species can only utilize bioluminescence.

The quantitative variation poses two kinds of challenges. When there is very little light, the amount of visual information it can carry is limited and must be used as efficiently as possible for the needs of the particular species (see below). In stronger light, on the other hand, it becomes a challenge for the photoreceptors to handle the overwhelming range of physical intensities. During evolution, this has implied heavy and changing selection pressures, as

vision has had to adjust to changing behavioural needs and different light environments on land and in water.

In animals with developed vision, light is collected by eyes that form an image on a mosaic of photoreceptor cells, reproducing to some degree of accuracy the spatial light distribution in the environment. The visual pigment molecules in the photoreceptor cells of the eye constitute the interface between the physical world of light and the physiological world of neural signalling. The more photons that are absorbed by visual pigments, the more information the neural image may potentially carry, and the finer may be the visual analysis in terms of spatio-temporal detail, discriminable contrast levels, movement and colour. However, photoreceptor cells (like neurons in general) are able to give graded responses only over ca. 3 log unit signaling ranges before saturating. This is exceeded by at least a billion-fold by the ranges of light intensities where animal vision may operate in nature.

Evolutionary adaptations to different light levels are evident already in eye morphology and optics. Eyes of night-active animals are generally large with a big pupil and short focal distance to maximize light collection and make the retinal image as bright as possible. Eyes for bright-light vision typically have a smaller aperture and a larger focal distance to favour a well-resolved retinal image at the expense of the brightness of the retinal image (see Walls 1942). Variation in pupil size can generally regulate the amount of light entering the eye by a factor of 10-20. The remaining huge variation must be handled by the photoreceptor cells and other neural layers of the retina (Warrant 1999). This is expressed in several evolutionary solutions. Most obvious is the duplex nature of most vertebrate retinas, implying that the stimulus range is partitioned between rods specialized for high sensitivity, and cones specialized for vision at higher light levels (see below). The basic sensitivity difference of rods and cones further correlates with different capacities for regulating sensitivity (light-adaptation), typically allowing rods to shift their intensity-response function over no more than 3 log units of mean light intensity, while cones have a virtually unlimited capacity to shift their operation range with increasing mean light intensity and thus retain the capacity to give graded responses to contrast around the mean level.

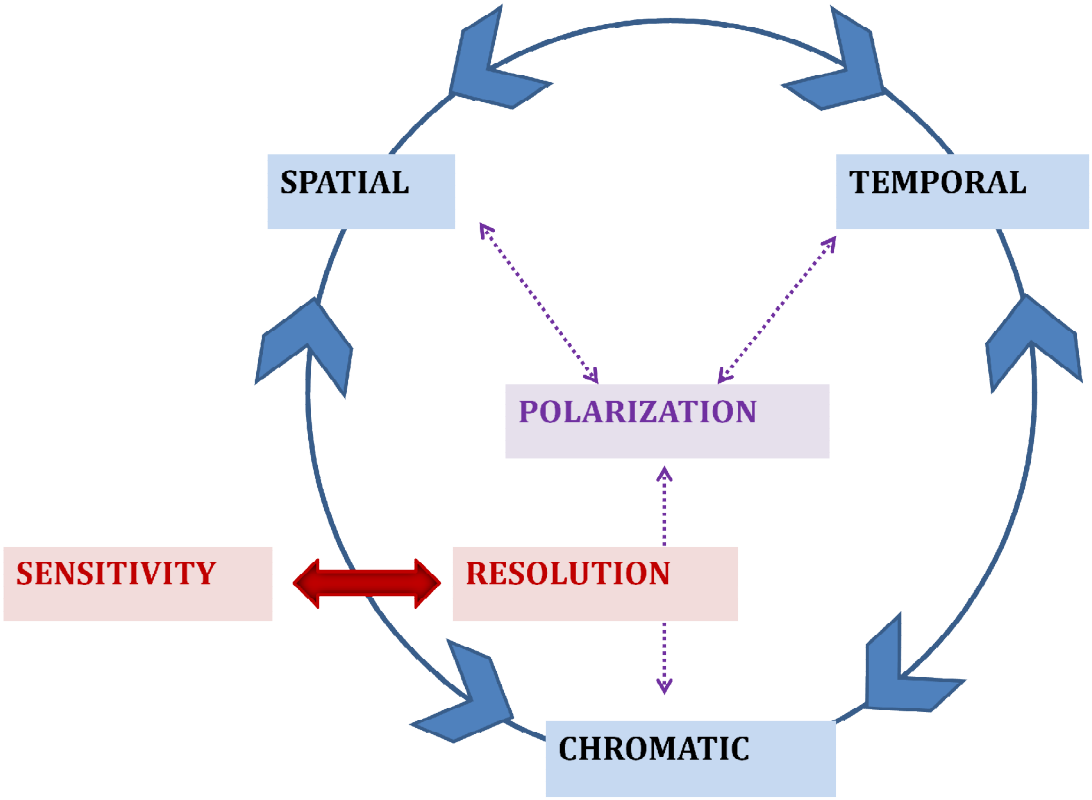


Fig. 2 A schematic representation of the trade-offs between different kinds of performance in animal vision. The quantity of light available determines the basic choice between extensive "pooling" for high light sensitivity and resolution of different aspects of the information carried by the light. Resolution may be differently allocated in several dimensions: spatial or temporal, or towards the resolution of the qualitative properties of light, wavelength (chromatic) resolution and, in some species, direction of polarization. Modified from Donner 1999.

The more light there is, the more information can it potentially carry. This may be utilized to support visual resolution in several different dimensions: for improved spatial acuity and/or temporal resolution, or for analyzing differences in wavelength distributions or polarization properties (in many animal groups, although not in mammals). It is impossible, however, to be good at everything at the same time. Animals have to make trade-offs between the different capacities according to their specific needs (see Fig. 2). Improvement in one respect occurs at the expense of others, either directly in the use of the photon flux, or by competing for costly investment in parallel neural pathways. The most basic trade-off is between sensitivity and resolution (Warrant 2004). Like in photography or technical image processing, pooling light in space (larger pixels) or time (longer exposures) improves reliability and thereby contrast sensitivity, while resolving the total photon flux into smaller portions (smaller pixels, shorter exposure times, or several colour channels) makes each of these noisier and less reliable. Thus diurnal animals that usually live in bright illumination may have invested in a dense mosaic of cones of several classes, enabling good acuity, movement

perception, and good colour vision at the expense of sensitivity. Nocturnal animals adapted to very low light intensities may have only one class of large photoreceptors optimized to collect sparse photons over long summation times (Lythgoe 1972; Bowmaker *et al.* 1991). In duplex retinas, a high rod/cone ratio is an obvious adaptation for high sensitivity (Crescitelli 1972; Locket 1977). Adaptations of aquatic animals living in deep or dark waters often parallel those seen in nocturnal land animals, maximizing sensitivity (absolute and achromatic contrast sensitivity) at the cost of low visual acuity, slow vision, and lack of wavelength discrimination.

The differences between dim-light vision and bright-light vision, between rods and cones, and between different rods and different cones, start in the visual pigments. This is the level investigated in the present thesis: which are the functional variables and constraints determining adaptation of visual pigments to different light environments and visual tasks.

1.2 PHOTORECEPTOR CELLS AND VISUAL PIGMENTS

1.2.1 RODS AND CONES

All vision starts with absorption of light by visual pigment molecules. In vertebrates, they are located in the outer segments of photoreceptor cells, rods and cones, in the distalmost part of the retina at the back of the eye.

As light enters the vertebrate eye, it passes through the cornea, the anterior and the posterior chamber, the lens, and the vitreous body to reach the retina. In the retina, it has to traverse several neural layers before reaching the rod and cone outer segments: the ganglion cell layer, the inner plexiform (synaptic) layer, the inner nuclear layer (with the cell bodies of amacrine cells, bipolar cells and horizontal cells), the outer plexiform (synaptic) layer, and the outer nuclear layer with photoreceptor cell bodies.

The visual pigments are membrane proteins packed in great concentration in the membranes of the outer segments. Rods and cones typically differ both by their morphology and functional properties. Rod outer segments are usually longer and their pigment molecules are mainly packed in the membranes of cylindrical discs that are separated from the plasma membrane. Cone outer segments are generally smaller and their pigment molecules reside in folds contiguous with the plasma membrane of a usually tapering outer segment. As an exception, some vertebrates may also have morphologically rod-like cones and cone-like rods (Goldsmith 1990). Functionally, rods have evolved to give large responses to each photon at low light levels. They have a limited capacity for light-adaptation and saturate in bright light. Cones are faster and less sensitive. In the dark-adapted state, the rod response to a single photon is 2 to 5 times slower than that of a cone, but

ca. 100 times larger (Yau 1994). Cones have a virtually limitless capacity to adapt to stronger mean illumination without saturating. They do this by decreasing gain and decreasing their pigment concentration (cf. Donner *et al.* 1998).

The two basic photoreceptor types also differ in that rods are usually of a single spectral class in a given retina, whereas cones come in several spectral classes. This enables wavelength discrimination and colour vision, which requires comparison of the outputs of at least two cone photoreceptor cells that differ with respect to their spectral sensitivities. Colour vision is almost ubiquitous among vertebrates. However, most mammals have poorer colour vision than fish, reptiles, birds or even amphibians, as most have only dichromatic vision (i.e., two cone visual pigments); typically blue and green or yellow (Jacobs 1983). Higher primates have regained trichromatic colour vision based on a secondary split in the green/yellow pigment some 30 million years ago in fruit-eating monkeys. This is believed to have facilitated the detection of fresh leaves and ripe fruits against green leaves (Dominy and Lucas 2001; Jacobs 2008). Some fishes living near the surface have even evolved tetrachromatic colour vision (Douglas and Partridge 1997), as have many birds and reptiles. Only a minority of vertebrate species are colour-blind (Yokoyama and Yokoyama 1996; Bowmaker 1991; Jacobs 1981, 1983).

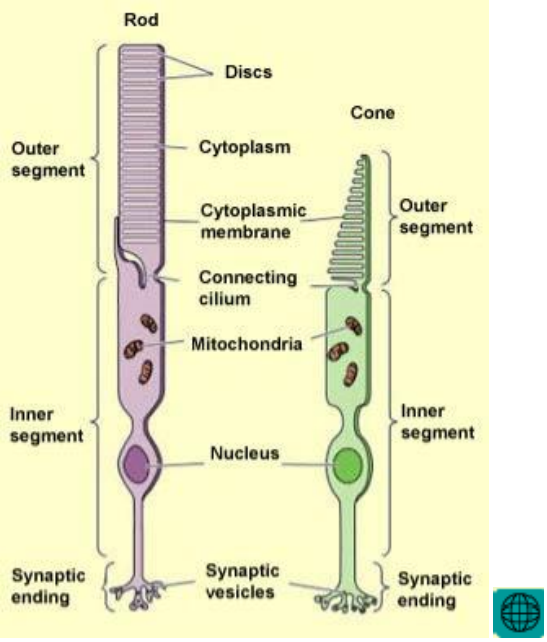


Fig. 3 Schematic picture of rod and cone photoreceptor. In photoreceptors, three different regions can usually be distinguished: 1. outer segment, where a stack of discs are embedded in the cell membrane, 2. inner segment, where the cell organelles lie, and 3. synaptic ending, from where the visual information is transmitted to the second-order neurons. (Image from http://thebrain.mcgill.ca/flash/d/d_02/d_02_m/d_02_m_vis/d_02_m_vis.html)

1.2.2 THE STRUCTURE OF VISUAL PIGMENTS

All visual pigments have a similar molecular structure, consisting of a protein called opsin to which a small chromophore (retinal), a derivative of vitamin A, is covalently bound. It is the chromophore that makes the molecule light sensitive. Opsin belongs to a large superfamily of integral membrane proteins, the G protein coupled receptors (GPCRs). It is a single polypeptide chain comprising 340-390 amino acids in the vertebrate opsin subfamily, which has been studied the most. Other subfamilies of opsin are less well known. Of the G_q-coupled opsin subfamily, dominant in arthropods and molluscs, squid opsin is the only well characterized member, with 448 amino acids and molecular weight 30-50 kDa (Terakita 2005; Murakami 2008).

Opsin is a typical 7-TM receptor protein with seven mostly hydrophobic α-helices traversing the membrane (Fig. 4). The N-terminal and three of the interhelical loops are in the intradiscal or extracellular space, while the intracellular parts (the C-terminal and the other three loops) in the cytoplasm are essential for G-protein activation by the activated pigment, and for shut-off by phosphorylation and arrestin binding. Opsin binds the chromophore's aldehyde terminus in the middle of its seventh transmembrane helix via a protonated Schiff's base at lysine^{296} (Bownds 1967; Ovchinnikov 1982; Hargrave *et al*. 1983, 1984). The positive charge of the Schiff base linkage is stabilized by a negatively charged counterion, glutamate^{113} in bovine rhodopsin (Zhukovsky and Oprian 1989; Sakmar *et al.* 1989) or glutamate^{180} in squid rhodopsin (Sekharan *et al.* 2010). Both lysine^{296} and glutamate$^{113/180}$ are highly conserved amino acid residues important in regulating the spectral absorbance and the stability of the opsin-bound chromophore. The structures of transmembrane helices and cytoplasmic loops are highly conserved, although the amino acid sequences may vary widely between different pigments.

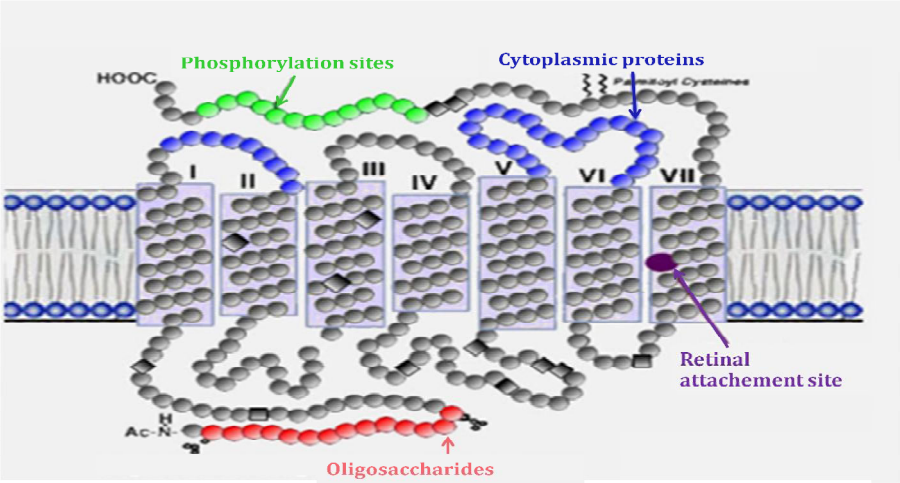


Fig. 4 Structural model of rhodopsin with seven transmembrane helices and the attachment site for retinal. Modified from Hargrave *et al.* 1984.

The chromophore (vitamin A aldehyde, called retinal) lies in the largely hydrophilic pocket formed inside the opsin. It is a conjugated chain of some 20 carbons with alternating single and double bonds and a β-ionone ring. In vertebrate visual pigments, two different kinds of retinals are used: 11-*cis* retinal (A1), whereby the visual pigments are known as rhodopsins and 11-*cis*-3,4-didehydroretinal (A2), whereby the pigments are known as porphyropsins. The functional difference between these chromophores is due to an extra double bond between the carbons at the positions 3 and 4 in the β-ionone ring. This extends the conjugated chain and thus favours absorption of longer wavelengths of light (lower photon energies). The drawback is decreased thermal stability (see further below) and decreased photosensitivity (see Bridges 1972).

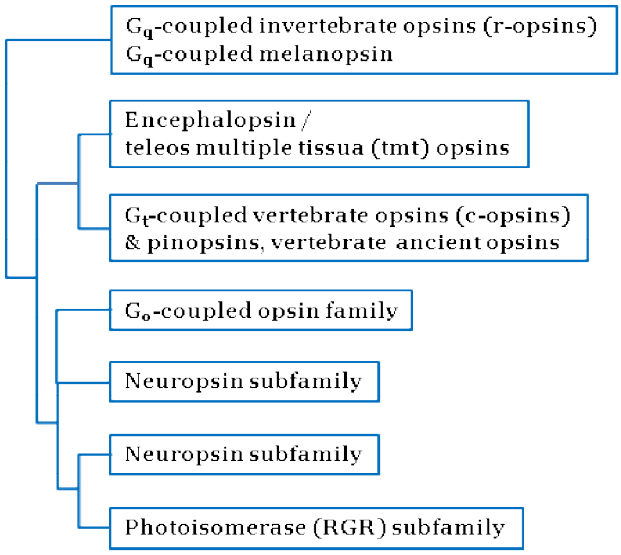
The properties of visual pigments can be adjusted by changing either of the two components, the protein or the chromophore. Fishes and amphibians as well as some reptiles may use either the A1 or the A2 chromophore, and in many species the proportions can be physiologically regulated, e.g., with season or different stages of life history (Dartnall *et al.* 1961; Tsin and Beatty 1980; Suzuki *et al*. 1985). Changes in the amino acid sequence of the opsin, on the other hand, underlie the evolution of visual pigments at both micro- and macro-levels, including the full evolutionary radiation of vertebrate opsins (see below). Opsin changes through mutations subsequently fixed by natural selection always require long (evolutionary) time scales. Yet, even phenotypic plasticity of vision may be based on opsin differences, as many fish species are able to regulate the relative expression levels of multiple pre-existing opsin genes in the same photoreceptor cells differentially during different stages of life history. For example, African cichlids express four different cone opsins to modulate their spectral sensitivities (Carlton and Kocher 2001; Fuller *et al.* 2003; Parry *et al.* 2005). They also express three other opsin genes but only during various points of their development. Conversely, the preservation of several functional opsin genes may allow rapid evolution in response to changing selection pressures (Spady *et al.* 2006).

1.2.3 PHYLOGENY AND CLASSIFICATION OF VISUAL PIGMENTS

The vertebrate opsin family consists of seven functionally different subfamilies according to molecular phylogeny (Fig. 5.A; Terakita 2005; Fernald 2006; Bailes 2007). It seems clear that the evolutionary diversification of subfamilies occurred early in animal evolution, in the hypothetical Urbilateria before the protostome-deuterostome split ca. 580 Mya (Lamb and Collins 2007). Five of these subfamilies form visual pigments. Vertebrate visual opsins are members of the ciliary opsin (c-opsin or G_t-coupled opsin) family. Photoreceptors with rod morphology usually express RH1 (460-530 nm) pigments, while the other four classes reside in

cone photoreceptors: SWS1 (355-440 nm), SWS2 (410-490 nm), RH2 (480-535 nm), and LWS (490-570 nm).

A



B

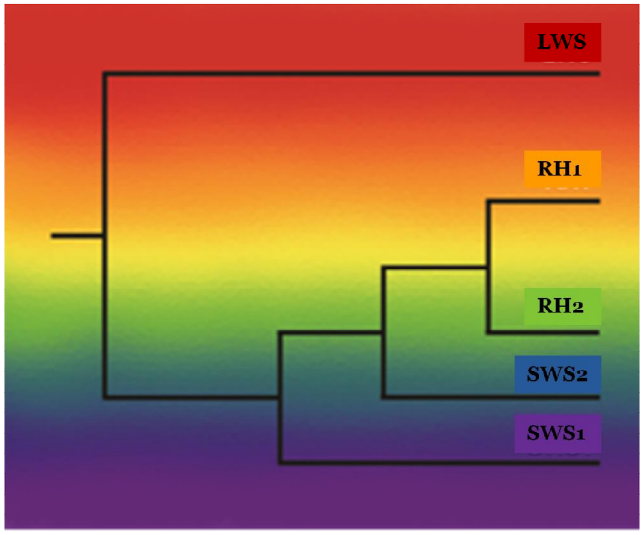


Fig. 5 Opsin phylogeny in Metazoa **(A)** and vertebrates **(B)**. **(A)** A schematic molecular phylogenetic tree inferred by the neighbour-joining method showing the seven known opsin subfamilies. Three families transduce light using G protein–coupled mechanisms (Gq, Gt, Go); Gq or r- (rhabdomeric) opsins are the predominant opsins in invertebrate photoreceptors and Gt or c- (ciliary) opsins in vertebrate photoreceptors. The function of encephalopsin and its teleost homolog tmt, which are found in multiple tissues, is unknown. Pinopsins, closely related to c-opsins, are expressed in the pineal organ of several vertebrates, and vertebrate ancient opsins are expressed in nonphotoreceptor retinal neurons, including amacrine and horizontal cells in teleost fish retinas. Similarly, neuropsins are found in the eye, brain, testes, and spinal cord in mouse and human, but little is known about them. Peropsins and the photoisomerase family of opsins bind all-trans-retinal, which is isomerized by light to the 11-*cis* form, suggesting a role in photopigment renewal. These are expressed in tissues adjacent to photoreceptors, consistent with this role. Modified from Fernald 2006. **(B)** Vertebrate opsins diverged into five subclasses. Modified from Bailes *et al.* 2007.

1.3 FUNCTIONAL PROPERTIES OF VISUAL PIGMENTS

1.3.1 SPECTRAL ABSORBANCE

As a photon hits the chromophore, it can cause an electron to be transferred from the ground state to an excited state of the molecule. This is possible if the energy difference between two different molecular orbitals falls within the same range as the energy (of some photons) of the incoming light. The absorbed energy achieves isomerization of the chromophore and activation of the pigment with a certain probability (called quantum efficiency), which has a practically constant value of ca. 2/3 across all vertebrate visual pigments studied (cf. Dartnall 1972). There is a change in the shape of molecule as it spins from the "bent" 11-*cis* to the "straight" all-*trans* configuration around the bond between the 11th and the 12th carbon atoms (Wald 1968). This in turn alters relative positions of the opsin transmembrane helices (Hubbard and Wald 1952; Wald 1968; Farrens *et al.* 1996; Park *et al*. 2008). As a result the cytoplasmic loops of the opsin become exposed for transducin activation, initiating the biochemical transduction cascade. The chain of reactions finally leads to closure of cation channels in the plasma membrane and thus to the electrical response (hyperpolarization) of the photoreceptor cell (Resek *et al.* 1993; Altenbach *et al.* 1996; Yang *et al.* 1996).

Each photon that activates a visual pigment molecule has the same effect regardless of the energy of the photon. This is called the principle of univariance (Naka and Ruhston 1966). In effect, a photoreceptor cell can signal only the number or rate of photons it catches, not their "colours".

Spectral absorbance is the most important functional variable of visual pigments. The absorbance spectrum, usually plotted as a function of the wavelength of light, describes the band of electromagnetic radiation accessible to the pigment. The spectra of visual pigments, even though derived from widely differing species, are basically similar, composed of several distinct absorption bands: the α-, β-, and γ-band etc. (Fig. 6). Of these, the α-band is the most interesting, because (together with the much weaker β-band) it reflects the electronic excitation of the opsin-bound chromophore (Jurkowitz *et al.* 1959). It forms a roughly gaussian curve describing the relative probability of each wavelength of light (i.e., photon energy) to be absorbed. The peak of the α-band is the wavelength of light absorbed with the highest probability (wavelength of peak absorbance, λ_{max}).

While the spectra of all visual pigments with the same chromophore have similar shape and can be made identical by transformations with λ_{max} as the only parameter (Dartnall 1953; McNichol 1986; Govardovskii *et al.* 2000), the parameter λ_{max} itself may take any value from ca. 350 nm to ca. 620 nm. This value depends on the two main components whose interaction determines the spectrum: the opsin and the chromophore. Changing the

chromophore from A1 to A2 in the same opsin shifts the peak towards longer wavelengths and changes the shape of spectrum, making it wider and lower (Bridges 1967, 1972). The dependence of λ_{max} on amino acid substitutions in the opsin is complex (see further below), although the spectral effects of a number of specific residues have been characterized.

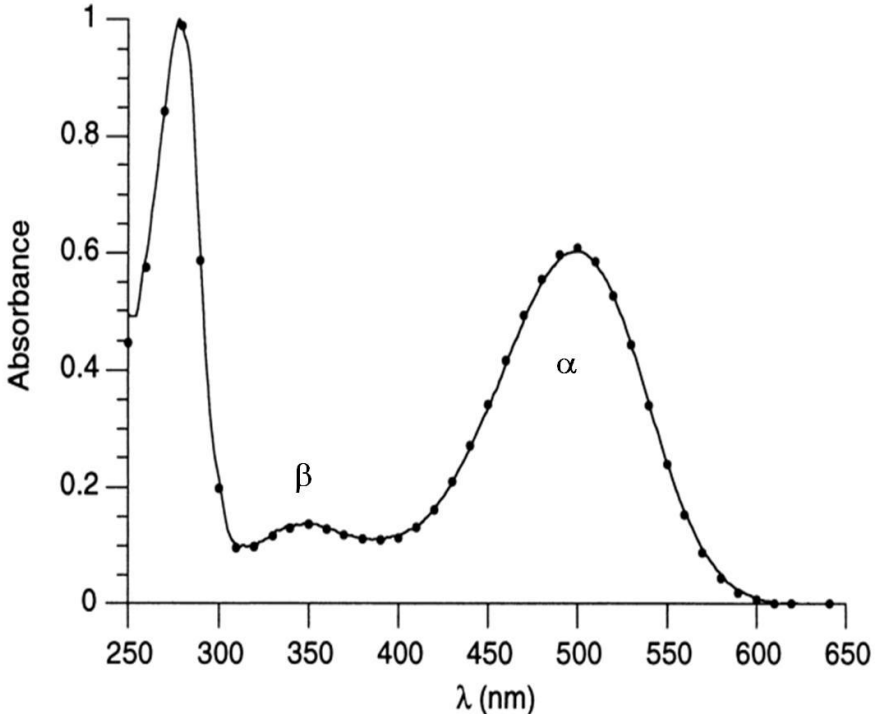


Fig. 6 Absorption spectrum of native bovine rhodopsin. The α- and β- bands correspond to absorption of light by the opsin-bound 11-*cis* chromophore (the β-band to absorption by part of its conjugated chain), and the γ-band and bands located at even shorter wavelengths (not shown) to light absorption by the protein. Modified from Eilers *et al.* 1999.

1.3.2 THE CONTRIBUTION OF THERMAL ENERGY TO THE ACTIVATION OF VISUAL PIGMENTS

The idea that thermal energy can supplement the energy of photons to achieve isomerization of the chromophore and activation of the pigment was first tested psychophysically more than 60 years ago. The Dutch physicist Hessel de Vries (1948) managed to record a subtle change in his own spectral sensitivity upon raising his body temperature in a bathtub. In pigment extracts where it is possible to alter temperature over wider ranges, significant shifts in λ_{max} and spectral shape were subsequently measured (St. George 1952; Yoshizawa 1972). At the same time, the English physicist William S. Stiles (1948) presented a model for thermal effects on spectral sensitivity. He suggested that the long-wavelength slope of absorbance spectra far beyond λ_{max} represents a domain where photon energy is insufficient to isomerize the chromophore, and activation is then achieved

only by a combination of energy from light and heat. If so, the relative spectral sensitivity of vision at very long wavelengths should decrease upon cooling and increase upon warming, as observed by de Vries. Stiles' model also suggests a method for determining the minimum energy needed for pigment activation, the photoactivation energy (E_a), from the temperature effect on spectral sensitivity in the long-wavelength domain (papers I, II).

1.3.3 THERMAL STABILITY AND NOISE

Even before the work of de Vries and Stiles (1948), it had been suggested that a molecule such as a visual pigment, which is designed to undergo a certain change when absorbing enough (photon) energy, may sometimes undergo the same change "spontaneously", due to thermal energy alone. It was also suggested that such spontaneous thermal events constitute an irreducible source of noise in the system (Autrum 1943; Barlow 1956). Therefore, high thermal stability of the visual pigment might be as important as high quantum catch for good vision in very dim light.

For vertebrate photoreceptors, noise attributable to thermal isomerizations was first reported by Denis Baylor and his colleagues in 1980 (Fig. 7) (Baylor *et al*. 1980, cf. however Yeandle 1958 about *Limulus* photoreceptors). In membrane current recordings from single toad rods in darkness, they noticed spontaneous discrete events ("bumps") that appeared identical to the responses to single photons they had described earlier (Baylor *et al.* 1979). Similar discrete "dark" events were soon recorded from macaque rods, suggesting that they are likely to occur in humans, too (Baylor *et al.* 1984). Several lines of evidence suggest that such events arise from thermal activation of single molecules of visual pigment (see, e.g., Firsov *et al*. 2002).

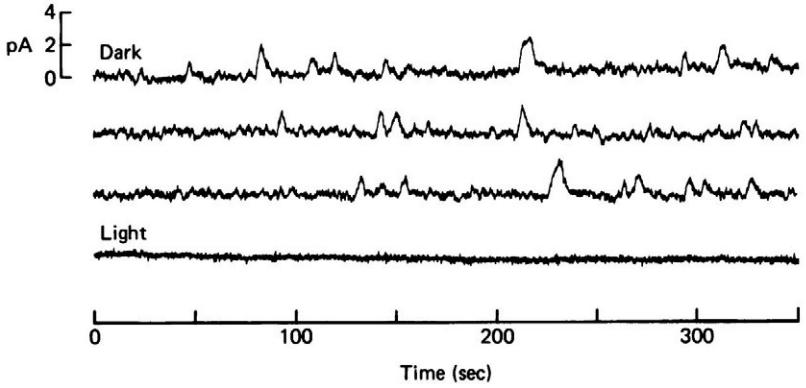


Fig. 7 Membrane currents from a single rod recorded first in total darkness (three top traces) and then under strong (saturating) light that shuts off all the light-sensitive current (bottom trace) (Baylor *et al.* 1980).

In rods, thermal dark events are rare. In a toad rod (containing 1-2 billions of pigment molecules) there is approximately one thermal activation per minute at 20°C. For the average rhodopsin molecule, this would correspond to an average "lifetime" of ca. 3000 years. In macaque rods, which have much smaller outer segments (implying smaller numbers of visual pigment molecules) but work at a higher temperature, the rate of thermal dark events per cell is about the same as in toad rods (Baylor 1980, 1984).

1.3.4 PIGMENT NOISE AND THE ABSOLUTE SENSITIVITY OF VISION

The random spontaneous activation of visual-pigment molecules by thermal energy constitutes a particular kind of noise ("shot noise") in the photoreceptor. As the resulting discrete "dark events" are identical to responses to single photons, there cannot even in principle be any way for subsequent stages in the visual system to tell the difference. Hence the background noise from thermal activations could set an ultimate limit to the absolute sensitivity of vision (the smallest number of photons that can be discriminated).

Detection of light or discrimination of contrast is a statistical task, which depends on the ratio of the mean signal to the standard deviation (SD) of random activity, noise, called the signal-to-noise ratio (SNR):

(1) $SNR = mean/SD$

In photoreceptors working in dim light, there are three main sources of random variation to consider when analyzing the statistics of detection of incremental light pulses. (1) Photon noise (quantal fluctuations) is connected with the physics of light itself: the number of photons actually received within a certain time window varies around a mean value according to a Poisson distribution. Three other major sources are intrinsic to the photoreceptor cell, namely, (2) discrete "dark" events due to thermal activation of visual pigment, and (3) "continuous" noise, thought to arise predominantly from thermal activation of phosphodiesterase (Baylor *et al.* 1980); (4) transduction noise causing variation in the amplitude and shape of the single quantum response (see Field *et al.* 2005).

The Poisson variation in the number of photons received is an unavoidable physical fact (de Vries 1948). The SD of the Poisson distribution is equal to the square root of the mean number N:

(2) $SD = \sqrt{N}$

The SNR of an "ideal observer" (with no intrinsic noise) detecting stimulus pulses that cause a mean number of "signal" photoactivations N_S in darkness is therefore:

(3) $SNR = mean/SD = N_S/(N_S)^{1/2} = (N_S)^{1/2} = \sqrt{N_S}$

If there is a background light present, photon noise will also include the variation in the "background" photoactivations N_B that may be confused with the stimulus:

(4) $SNR = N_S/(N_S + N_B)^{½}$

The randomly occurring discrete dark events due to thermal pigment activations are identical to photon events N_S and N_B. If the number of dark events occurring in the same time window is D, we get:

(5) $SNR = N_S/(N_S + N_B + D)^{½}$

There is no way of selectively "removing" D. By contrast, other types of noise having clearly lower amplitude (as is true of "continuous noise" in many species of dark-adapted rods) or different frequency characteristics compared with photon responses may be reduced or eliminated by filtering or thresholding (see, e.g., Field *et al*. 2005).

From eqn (4) it is obvious that pigment noise (D) becomes unimportant when there is much light ($N_S + N_B >> D$). Even in the strictly dark-adapted situation, there are many physiological caveats related to the fact that there is huge variation in the rates of spontaneous rod events between photoreceptor species and that the continuous noise may be so close in amplitude to single-photon responses that the two cannot be efficiently separated e.g. by thresholding nonlinearities. Regardless of all this, it remains a fact that high rates of thermal activation of visual pigment (large D) will necessarily set one absolute limit to the number of "stimulus" photons that the system can detect in darkness. Therefore, the thermal stability (or conversely: the propensity to produce "false", thermal responses) may be considered as a second crucial functional variable related to the activation of visual pigments, next to spectral absorbance.

1.3.5 THE RELATION BETWEEN SPECTRAL SENSITIVITY, ACTIVATION ENERGY AND THERMAL STABILITY OF VISUAL PIGMENTS

High visual sensitivity (high SNR) requires catching photons efficiently but having low noise. Photon catch depends on how well the spectral absorbance of the pigment is "matched" to the spectral distribution of light in the environment. On the other hand, thermal activation of rod pigment appears to be the dominant source of noise limiting absolute sensitivity in many species, including humans and toads (Barlow 1956; Aho *et al*. 1988; Donner 1992). Thus, both signal and noise at absolute threshold reflect the properties of the visual pigment. As explained above, there are simple physical reasons to hypothesize that the spectral absorbance and thermal stability are not independent properties of a visual pigment: red-tuning means that the pigment can be activated by photons of lower energies; hence it may be expected that it is also more susceptible to activation by heat. This was the reasoning in Horace Barlow's seminal article "Purkinje shift and retinal noise" (1957). Barlow suggested that the ubiquitous blue-shift of night vision compared with day vision is prompted by the need to minimize thermal noise in dim-light vision. Night illumination (moonlight and starlight) is in fact red-shifted compared with daylight.

The hypothesized dependences between the properties of visual pigments are shown schematically in Fig. 8: the energy needed for activation of the pigment (E_a) is inversely related both to λ_{max} and to the probability of thermal activation. Therefore increasing λ_{max} is predicted to correlate with increased thermal noise. Tuning a pigment for good performance in an environment dominated by long-wavelength light will be an optimization task where SNR gain by increasing photon catch must be balanced against SNR loss due to increasing thermal noise.

The theoretical relationship schematically depicted in Fig. 8 defines the core hypothesis studied in the first part of this thesis. When studies I and II were carried out, the experimental evidence was sparse. The main purpose of these studies was to test the left part of the scheme: the relation between absorbance spectra and activation energy. For the experimental work, we selected a few model photoreceptor species to cover some critical points in the variation space.

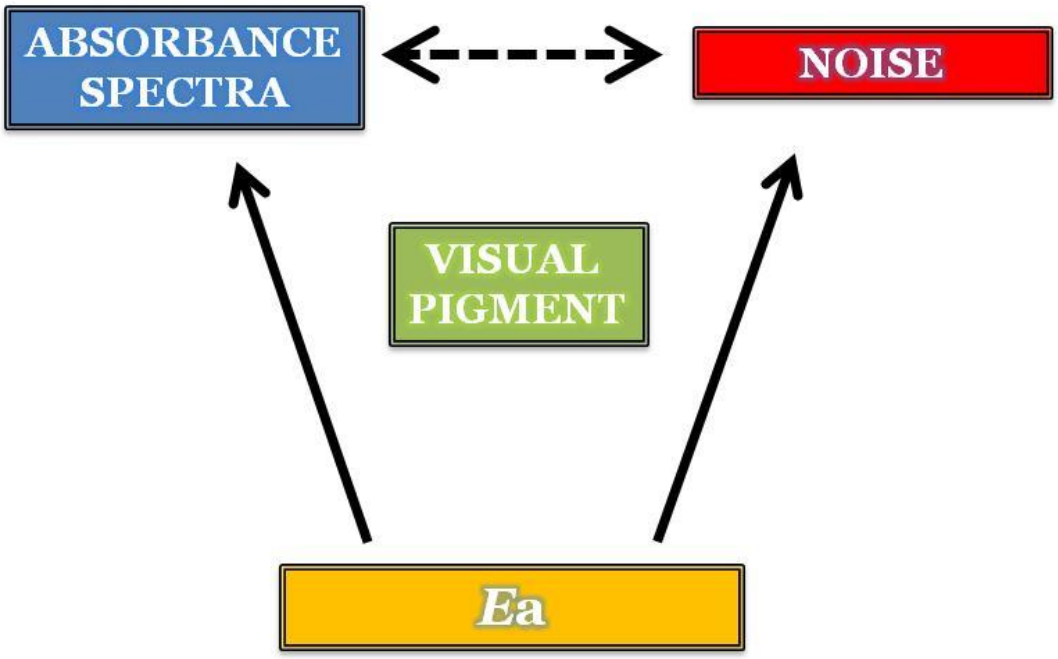


Fig. 8 The hypothesized relation between the main functional variables of visual pigments that motivated studies I and II of the present thesis.

1.3.6 BLEACH AND REGENERATION

After activation, a visual pigment molecule cannot serve photoreception until the all-*trans* chromophore has been removed from the opsin and the pigment has been regenerated by introduction of a new 11-*cis* chromophore. The whole process from activation (bleach) to regeneration is referred to as the visual cycle. In chromophore recycling, the retinal pigment epithelium (RPE) plays a central role and was for long thought to be the only source of 11-*cis* delivery to photoreceptors. A recently characterized Müller-cell pathway (Wang and Kefalov 2011), however, is important for cones and may underlie the differential (A1/A2) chromophore delivery to rods and cones found in paper *III*.

The time to activate the Rh is 200 fsec (Kandori *et al.* 2001) and the lifetime of activated rhodopsin (Rh*) is some 50 ms (Burns and Pugh 2009), after which it decays into an inactive form, which causes the Schiff-base to hydrolyze (Okada *et al.* 2001). As a result the all-*trans* retinal is released from the opsin and reduced to all-*trans* retinol (=vitamin A) on a time scale of minutes in rods (Shichida *et al.* 1994). Fig. 9 shows the visual cycle through the RPE. The all-*trans* retinol might be transported to the RPE by an interreceptor retinoid binding protein (IRBP) (Bunt-Milam and Saari 1983; Okajima *et al*. 1994; Ala-Laurila *et al.* 2006; Wu *et al.* 2007) although Kolesnikov *et al.* (2011) found no evidence for the role of IRBP in transporting chromophore. In RPE, at least three enzymes are associated

with the regeneration of all-*trans* retinol to 11-*cis* retinal. The 11-*cis* retinal is similarly transported to the photoreceptor outer segments where it can bind with opsin to regenerate functional visual pigment. When significant amounts of visual pigments are bleached it may take up to 30–60 minutes to restore the dark-adapted state of a rod, with correspondingly slow recovery of rod-mediated visual sensitivity (Hecht *et al.* 1937; Aguilar and Stiles 1954; Campbell and Rushton 1955).

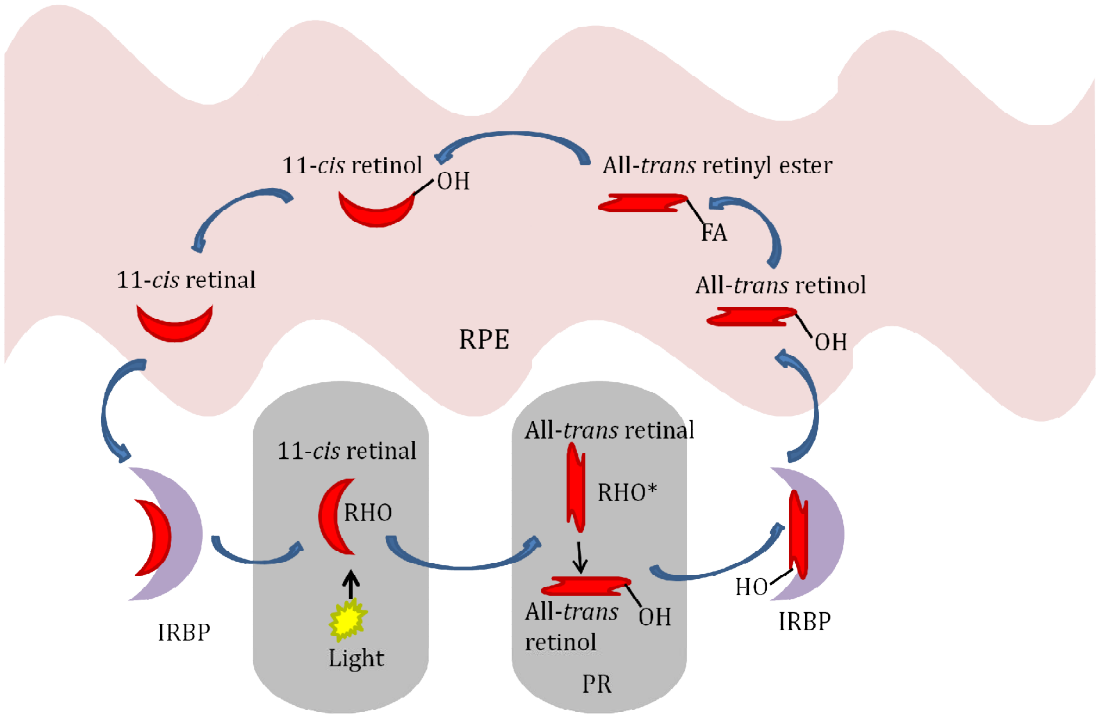


Fig. 9 The visual cycle through the RPE. All-*trans*-retinal is reduced by all-*trans*-retinol dehydrogenase to all-*trans*-retinol in the rod outer segment, all-*trans*-retinol is bound to IRBP and transferred to the retinal pigment epithelium (RPE). There, it is esterified by retinol acyltransferase to all-*trans*-retinyl ester (a fatty acid), which is isomerized and hydrolyzed by isomerohydrolase RPE65 to 11-*cis*-retinol. 11-*cis*-retinol dehydrogenase oxidizes 11-*cis*-retinol to 11-*cis*-retinal (at the same time bound to cellular retinaldehyde binding protein CRALBP). As 11-*cis*-retinal is transferred through the extracellular space to rod outer segment it might be bound to IRBP. Modified from Wright *et al.* 2010.

It was long believed that both rods and cones were wholly dependent on the same RPE visual cycle (Fain *et al.* 1996; Reuter *et al.* 1971). Yet, cones are specialized for functioning in photopic conditions and require greater amounts of 11-*cis* retinal than rods do. At light exposure, rod and cone pigments bleach at equal rates (Hárosi 1975) but after a bright exposure, cones recover within 3-4 minutes. As chromophore supply to the photoreceptors is the rate-limiting step in regeneration (Lamb and Pugh 2004), it was suggested that there might also exist a second, cone-specific

visual cycle (Mata *et al.* 2002). Both biochemical and physiological evidence of this cycle have now been found, although many details are still uncertain (see Wang and Kefalov 2011).

Cone all-*trans* retinol may either enter the classical RPE visual cycle or be transported to Müller cells, which stretch from ganglion cells to the outer limiting membrane and reach only the inner segment of photoreceptors. When all-*trans* retinol is transferred to Müller cells, it binds to chromophore-binding-protein and is isomerized to 11-*cis* retinol by isomerase II (Bunt-Miliam and Saari 1982; Eisenfeld *et al.* 1985; Mata *et al.* 2002, 2005). Then it is transferred to the cone inner segment, maybe via IRBP (Crouch *et al*. 1992; Jin *et al.* 2009; Parker *et al.* 2009) and oxidized by an unknown enzyme to 11-*cis* retinal for pigment formation (Jones *et al.* 1989).

RPE and cone-specific visual cycles are thought to work simultaneously. Even if both cycles would work at the same rate, the effective doubling of the amount of chromophore results in 4-fold increase in cone sensitivity compared to RPE cycle working alone (Wang and Kefalov 2011). It is the cone-specific visual cycle that makes possible extension of the functional range of cones to photopic light levels (Wang and Kefalov 2011). In the present thesis, it is speculated that these two mechanisms working side by side can also enable independent spectral tuning of rod and cone visual pigments by differential chromophore use, which can have a huge impact on tuning of animal vision to different environmental illuminations (paper *III*).

1.4 MOLECULAR MECHANISMS FOR TUNING VISUAL PIGMENTS

1.4.1 OPSIN TUNING

In an evolutionary perspective, most spectral tuning of vision depends on mutations in the amino acid sequence of the opsin protein (Yokoyama 2002). Fixation of such changes usually takes at least 10^3-10^4 generations (Jokela-Määttä *et al.* 2007; Larmuseau *et al.* 2010). The mutations must generally occur around the chromophore pocket to affect the electrostatic or steric environment of the chromophore and thus the energy gap between the chromophore ground and excited states (Hunt 2001). At best, a single amino acid substitution can shift λ_{max} by even 75 nm (Shi *et al.* 2001), but tuning is generally implemented by several substitutions, each with a comparatively small effect.

Virtually indistinguishable absorbance spectra can be realized by widely differing opsin sequences (see, e.g., discussion in Jokela-Määttä *et al*. 2009). This also suggests the possibility that the hypothetical correlation triangle

shown in Fig. 8 may not be incompatible with significant freedom for independent tuning of λ_{max} and thermal stability (cf. Ala-Laurila *et al*. 2004b). Fyhrquist *et al.* (1998a) looked for amino acid substitutions that might specifically increase thermal stability by sequencing pigments from toad and frog rods with similar absorbance spectra but different noise properties. Due to the fairly large number of differences found, however, the results remained inconclusive. By contrast, a recent study by Luo *et al*. (2011) gives support to stricter correlations more strongly constrained by basic physics.

1.4.2 TUNING BY CHROMOPHORE

Chromophore shifts (in vertebrates A1 ↔ A2) enable fast shifts in spectral sensitivity, e.g. with season. The mechanism is unavailable to "warm-blooded" vertebrates (birds and mammals), arguably because the A2 chromophore would be too noisy at body temperatures of 35-40°C. In known A1 pigments, λ_{max} varies approximately from 350 to 584 nm and in A2 pigments approximately from 400 to 635 nm (Dartnall and Lythgoe 1965; Kleinschmidt and Hárosi 1992; Archer 1999; Britt *et al.* 2001). The A1-A2 system may generally be interpreted as a means of ensuring flexible matching of spectral sensitivity to the predominantly longer-wavelength light spectra in coastal and fresh waters (Lythgoe 1972). Replacing A1 by A2 in the same opsin red-shifts and broadens the absorption spectrum and decreases the photosensitivity of the visual pigment (Dartnall and Lythgoe 1965; Whitmore and Bowmaker 1989; Hárosi 1994; cf. Dartnall 1972; Bridges 1972). Many amphibians and fishes shift between A1 and A2 with season or developmental stage (Beatty 1984; Lythgoe 1984; Whitmore and Bowmaker 1989; Hárosi 1994). For example, the frog genus *Rana* shifts from A2 to A1 during metamorphosis from aquatic tadpoles to terrestrial adults (Wald 1946; Liebman and Entine 1968; Reuter 1969), while the clawed frog *Xenopus laevis* has a mixture of A1 and A2 chromophore as a tadpole but changes gradually to mainly A2 as a fully aquatic adult (Crescitelli 1973).

The seasonal effects may depend on changes in light period, light intensity, or temperature (Bridges 1965, Allen and McFarland 1973). The changes are hormonally regulated (Beatty 1969; Beatty 1984; Temple *et al*. 2008). The tendency towards use of A2 during winter has been interpreted as a response to changes in the surrounding illumination (Knowles and Dartnall 1977), but is also consistent with the lesser "cost" of using the noisy A2 chromophore at lower temperatures (cf. Donner *et al.* 1990). Many fishes (e.g., salmon) change from A1 to A2 as they migrate from the ocean to a river to spawn (Allen *et al.* 1973; Temple *et al*. 2006). In general, marine species utilize more A1 chromophore and freshwater species A2 chromophore, broadly correlating with spectral differences in the respective habitats. There are also many animals having both A1 and A2 at the same time. One of these

is the American bullfrog *Rana catesbeiana*, the rods of which are studied in the present thesis (paper II). The adult bullfrog has A2 at the dorsal rim of the retina and A1 in the ventral part (Reuter *et al*. 1971), in proportions somewhat depending on season. This may be correlated with the different fields of view of the parts when the frog lies at the water surface in a pond: underwater views project to the dorsal part, the sky overhead to the ventral part. Yet, many fishes and amphibians and most reptiles (like birds and mammals) possess only A1. For example, the genus *Bufo* has only A1 although its behaviour is similar to *Rana* in many respects (Peskin 1957; Partridge *et al.* 1992).

The effect of the A1 → A2 shift on λ_{max} increases with increasing λ_{max} of the A1 pigment over most of the range and can be 50 nm or more for the most long-wavelength sensitive pigments (Hárosi 1994; Whitmore and Bowmaker 1989). The shift is caused by the extra double bond in the cyclohexane ring of A2 retinal, which extends the conjugated π-system of the polyene chain. This modification of the molecule requires the enzyme retinol dehydrogenase (Provencio and Foster 1993). The two sources (RPE and Müller cells) supplying 11-*cis* chromophore to rods and cones might potentially deliver different proportions of A1 and A2, although it is not known if the Müller cells contain the enzyme for the A1 → A2 conversion.

2 AIMS OF THE STUDY

Part 1

The overarching aim of the first part (studies I and II) was to test the scheme shown in Fig. 8, which was largely hypothetical when the studies were carried out more than ten years ago. They were among the first in a line of research that has since firmly established the fundamental correlations between increasing λ_{max}, decreasing activation energy, and increasing thermal noise.

The specific aims were :

Study I
To determine the minimum energy needed for photoactivation (E_a) of three A1 visual pigments from temperature effects on the long-wavelength slope of photoreceptor sensitivity spectra (Srebro 1966; Koskelainen *et al.* 2000). A well-characterized "reference" photoreceptor, the rhodopsin rod of the toad *Bufo marinus*, was compared with (i) the spectrally *different* "green" rod of the same species; (ii) the spectrally and thermally *similar* rhodopsin rod of *Bufo bufo*. In both toad species, the rates of thermal dark events as well as the amino acid sequence of the "red" rod opsins are known (Baylor *et al.* 1980; Fyhrquist *et al.* 1998a, b; Firsov *et al.* 2002).

Study II
To study the relation between λ_{max} and E_a in A2 pigments and the effect of the A1 → A2 chromophore shift. The photoreceptors were selected to allow comparisons between (i) two rod pigments having the same opsin but different chromophores, (ii) two A2 rod pigments having similar λ_{max} values, and 3) two A2 pigments having different λ_{max} values.

Part 2

The second part of the thesis (study III) represents an ecological / evolutionary application of the insights to which studies I and II had contributed regarding effects of chromophore use and λ_{max} on visual signal-to-noise ratios. The initial aim was to look generally for differential evolutionary adaptations of the visual pigments in populations of a model species, the nine-spined stickleback (*Pungitius pungitius*), which had been isolated in different water bodies since the last glaciation. Finally, the study became focussed on the discovery of (genetically determined) differences in chromophore use between rods and cones of the different populations and understanding the implications of these differences.

3 MATERIALS AND METHODS

3.1 ANIMALS AND VISUAL PIGMENTS STUDIED

Table 1 List of animal species and visual pigments studied, and temperatures used in papers I and II.

Species	**Photoreceptors**	**Chromophore**	**λ_{max} (20˚C)**	**MSP (°C)**	**ERG (°C)**
Bufo bufo	rod	A1	503 nm	8,5; 28,5	8,5; 28,5
Bufo marinus	red rod	A1	504 nm	0; 8,5; 28,5; 40	8,5; 28,5
	"green" rod	A1	433 nm	0; 8,5; 28,5; 40	-
Rana catesbeiana	rod (ventral retina)	A1	502 nm	8,5; 28,5	8,5; 28,5
	rod (dorsal retina)	A2	525 nm	8,5; 21; 28,5	8,5; 28,5
Carassius carassius	rod	A2	526 nm	8,5; 28,5	8,5; 28,5
	L-cone	A2	619 nm	21,0	5,0; 15,0; 25,0

The species used in papers I and II are listed in Table 1. *Bufo marinus* and *Rana catesbeiana* were provided by commercial suppliers and *Bufo bufo* and *Carassius carassius* were caught in the wild. Toads and frogs were kept at room temperature in 12h / 12h cycle, and fed with appropriate food, except *Bufo bufo* which were kept unfed in hibernating conditions (at 5˚C). The fishes were kept in aquaria in RT and used shortly after catching. *Rana catesbeiana* were kept in special light and temperature conditions which favour the formation of either A1 or A2 pigment (cf. II, Reuter *et al.* 1971; Donner *et al.* 1990).

Nine-spined sticklebacks (*Pungitius pungitius*) were caught with fishing nets and transferred in tanks to aquaria were they were kept at approximately 15 ˚C in 12h / 12h cycle and provided with appropriate food (cf. III). All fish studied were kept in standardized conditions for more than half a year before recordings were carried out. Locations of the habitats of the eight populations studied were: Abbortjärn (ABB) and Bölesviken (BOL) in Sweden, Helsinki (HEL), Iso-Porontima (ISO), Pyöreälampi (PYO) and Rytilampi (RYT) in Finland, and Mashinnoye (MAS) and Levin Navolok

(WHI) at the White Sea in Russia. Sea habitats are BOL, HEL, and WHI. ISO is a lake habitat, and the rest are ponds.

3.2 METHODS AND DATA ANALYSIS

Here, only a brief summary is given. For details, the reader is referred to the original publications.

3.2.1 MEASUREMENT OF PIGMENT ABSORBANCE SPECTRA BY MICROSPECTROPHOTOMETRY (MSP) IN ISOLATED PHOTORECEPTORS *(I-III)*

Absorbance spectra of isolated photoreceptor outer segments were measured (350-790 nm) with a single-beam, computer controlled, fast scanning MSP (Govardovskii and Zueva 1988; Govardovskii *et al.* 2000; Ala-Laurila *et al.* 2002).

The animals were dark-adapted, usually overnight. The animal to be used was killed by decapitation, the brain and spinal cord were destroyed with a needle, and the eyes were enucleated under a preparation microscope in dim red light (λ>650 nm). In 2011 (paper III), all preparations were done under infra-red LED light (peak emission at 850 nm and 50 nm half-bandwidth) with the aid of an IR-viewer to protect especially the L-cone pigment from bleaching. The eyes were hemisected and the retinas were carefully isolated. A small piece of one retina was teased apart in a drop of Ringer, and the sample was covered with a coverslip sealed at the edges and placed on the microscope stage for recordings.

After recording, the raw absorbance spectra of single cells were averaged within individuals so that data from every animal were analyzed separately. A Govardovskii *et al.* (2000) template (A1, A2 or mixed) was fitted to the α-band of the spectrum. In the fitting program, λ_{max} of A1 and A2 versions of a pigment are coupled by Hárosi's (1994) improved formula for the relation first described by Dartnall and Lythgoe (1965). Since A1 and A2 pigment spectra have different shapes, the shape of the spectrum may be used as a clue to chromophore identity. For a chromophore mixture (A1/A2), the ratio is obtained from the proportions of A1 and A2 templates in the best-fitting sum. In many cases, an independent estimate of λ_{max} was desirable in order to check for different kinds of systematic errors connected with template fitting. Then least-squares second-order polynomials were fitted to data points around the peak of the spectrum. The estimates of λ_{max} thus obtained are practically independent of those from template-fitting, since the latter method relies almost entirely on the sloping parts of spectra. For A1/A2 mixtures, it is possible to determine chromophore proportions from λ_{max} alone, if λ_{max} of either the pure A1 or pure A2 component is known.

3.2.2 MEASUREMENT OF THE SPECTRAL SENSITIVITY OF PHOTORECEPTORS BY TRANSRETINAL ELECTRORETINOGRAM (ERG) (*I, II*)

Spectral sensitivities of rods and cones were measured by ERG across dark-adapted isolated retinas where synaptic transmission had been blocked by sodium-L-aspartate and glial (Müller-cell) components by $BaCl_2$ (Donner *et al*. 1988; Heikkinen *et al*. 2009). Photoresponses were recorded by Ag/AgCl electrodes placed at opposite sides of the retina. The transretinal signal recorded is the ohmic voltage generated by the massed photocurrents of all photoreceptors and by possibly remaining secondary currents from other retinal cells. The components from different photoreceptor types can be isolated fairly reliably by appropriate protocols for stimulation and analysis (see Koskelainen *et al.* 1994, 2000; Donner *et al.* 1998).

Spectra covered the range 434 – 777 nm in wavelength steps varying across the spectrum depending on the precision needed. For each measurement wavelength, generalized Michaelis functions were fitted to intensity-response data recorded at 4-5 flash intensities. Relative spectral sensitivities were determined from the lateral shifts of the I-R functions compared to that at a reference wavelength near peak. Recordings at the reference wavelength were repeatedly interleaved between recordings at 3 to 5 test wavelengths, allowing correction even of minor general drifts in sensitivity.

3.2.3 DETERMINATION OF THE ENERGY FOR PHOTOACTIVATION (E_A) OF THE VISUAL PIGMENT (*I, II*)

According to theory of Stiles (1948), further developed by St. George (1952) and Lewis (1955), the photoactivation energy (E_a) of visual pigments can be determined from temperature effects on spectral sensitivity in the long-wavelength range. In the far red end of the spectrum, the energy of a photon is too low to excite the visual pigment molecule, but when supplemented with thermal energy (heat), it may be sufficient to effect the transition from the ground state to the first excited state. Thus, warming the pigment will increase the relative spectral sensitivity at very long wavelengths and the effect is stronger and starts at shorter wavelengths the higher the pigment's E_a is. The size of temperature effect allows estimation of the photoactivation energy. The methods, including all the potential error sources that must be observed, are described in detail in paper (I).

MSP and ERG spectra were "glued together" for determination of E_a. It may seem an odd idea to combine two kinds of spectra obtained by two quite different techniques, but it was necessary, as neither is good enough alone over the entire wavelength range needed. MSP is excellent in the main absorbance band where pigment absorbance is relatively high, providing high

quality data in short times. At low absorbances, however (e.g., at long wavelengths), MSP provides no useful signal. By contrast, only the power of the light source sets a long-wavelength limit to spectral sensitivities that can profitably be measured by electrophysiology. The numbers of pigment activations in the photoreceptors can always be increased by increasing flash intensity, and the molecular amplification in photoreceptors provides measurable electrical responses even to rather few pigment activations. The ERG technique has the additional bonus of averaging responses from thousands of photoreceptors, giving even dim-flash responses a good SNR. On the other hand, ERG data around peak is problematic because of flattening of the spectrum due to "self-screening" in the outer segments of photoreceptors under longitudinal incidence of the light (Dartnall and Goodeve 1937; Alpern *et al*. 1987), and reliable purification of responses from a single photoreceptor type in a mass signal may be difficult. To get reliable spectra, MSP and ERG data were therefore joined together so that the best parts of both were utilized for the E_a determination (see paper I).

3.2.4 LIGHT MEASUREMENTS (*III*)

The spectral distribution of downwelling light (quanta m^{-2} s^{-1} nm^{-1}) was measured in the habitats of four Fennoscandian populations of nine-spined sticklebacks (*Pungitius pungitius*) over the interval 400-750 nm using a QSM 2500 submersible quantum spectrometer (Techtum, Umeå, Sweden). The device is based on a light meter and a filter, which with the aid of step motor scans the whole spectral range in approximately one minute. Measurements were performed at the water surface and at 1-5 meter intervals below surface (minimum of two scans at every depth) until the bottom was reached. The measurements were done in September 2011 between 11 AM and 3 PM, during the brightest time of the day.

3.2.5 OPSIN SEQUENCING (*III*)

The gene coding the opsin part of the rod visual pigment was sequenced in all the eight populations of the nine-spined sticklebacks to find out possible variation in coding sequences. Total genomic DNA of three individuals from each population was extracted using NaOH-boiling (Duan and Fuerst 2001). Teleost primers were designed for nested polymerase chain reactions (PCR) to amplify the fragments of gene that corresponded to parts of the opsin transmembrane. The protocol is described in detail in paper III.

3.2.6 PRINCIPAL COMPONENT ANALYSIS (PCA) (*III*)

By PCA it is possible to divide mixed pigment spectra into components that explain certain fractions of the total variance (Ward *et al.* 2003; Mäkelä *et al.* 2011; Cuthill *et al.* 1999). In this thesis PCA was applied to stickleback rod and M-cone spectra to find differences in the composition of spectra at the population level. The components were plotted and primary component graphs with clear peaks were recognized. The peaks were characterized by least-squares fitting of second-order polynomials (see paper III).

4 RESULTS

4.1 THE ACTIVATION ENERGY OF VISUAL PIGMENTS STUDIED BY TEMPERATURE EFFECTS ON ABSORBANCE SPECTRA: DEPENDENCE ON λ_{MAX} AND CHROMOPHORE (*I, II*)

The effect of temperature change on absorbance spectra was mainly used as a means for measuring E_a according to the hypothesis derived from Stiles (see above) and relating it to λ_{max}. Besides producing data for this main calculation, however, the experiments yielded a number of observations on shifts in λ_{max} and changes in shape around peak. These results may be interesting from other viewpoints, and are also noted below, although only those related to the main purpose (E_a) will be given more extensive discussion. The photoreceptors studied were of seven kinds, selected to allow as many relevant comparisons as possible. The photoreceptors together with experimental temperatures (″warm″ and ″cold″), are given in Table 1.

4.1.1 VARYING ACTIVATION ENERGIES IN RODS WITH A1 CHROMOPHORE AND SIMILAR ABSORBANCE SPECTRA

Our ″reference″ A1 pigment, that of *Bufo marinu*s red rods, was studied at 8.5 (″cold″) and 28.5 °C (″warm″) both by ERG and MSP (paper I). Warm MSP spectra were well-fitted by Govardovskii *et al*. (2000) A1 templates, which gave λ_{max} = 503.9 nm. Cold MSP spectra were shifted and skewed towards the red, and the template fits were less beautiful (estimated λ_{max} was 504.6 nm). Judged by second-order polynomials (parabolas) fitted to the peaks (points ≥ 0,9 of maximum absorbance) the temperature change shifted λ_{max} from 503.1 nm (warm) to 504.2 nm (cold).

To get a more precise picture of the temperature effects, *B. marinus* rhodopsin was studied by MSP at two additional temperatures (see Table 1). The temperature dependence of λ_{max} was estimated to be -0.06 nm/˚C as calculated from the long-wave slope of MSP spectra. As the slope showed a slight but systematic curvature, a second-order polynomial was also fitted to it ($y = -0.00056x^2 - 0.039x + 504.6$). The change in *B. marinus* λ_{max} with temperature was similar to that of *Rana catesbeiana* rhodopsin as measured in solution over a wide temperature range (-100˚C - +23˚C) (St George 1952). Both these amphibian rhodopsins, on the other hand, were found to change more steeply than bovine rhodopsin (i.e., rhodopsin from a “warm-blooded” species). The differences could not be explained by the difference in

physiological temperatures and indicate real differences between A1 rod pigments with rather similar absorbance spectra.

At very long wavelengths, the relative sensitivity of *B. marinus* red rods was found to be higher in warm spectra than in cold spectra, in agreement with Stiles' hypothesis. The cold/warm slope ratio was 1.684/1.570 = 1.073 (= 76% of the theoretical prediction according to Stiles' hypothesis), and the inverse ratio of the experimental temperatures was 301.7K/281.7K =1.071.

Similar experiments were done with *B. bufo* rods (paper I), which are very similar to *B. marinus* rods in both spectral and noise properties (Fyhrquist *et al*. 1998a; Firsov *et al*. 2002). The λ_{max} values obtained were 501.9 nm (warm) and 503.0 nm (cold). The slope of the spectrum at very long wavelengths was 1.522 x 10^{-5} m in warm and 1.636 x 10^{-5} m in cold. The slopes were a bit shallower than in *B. marinus* but the ratio between curves was still almost the same, 1.075.

The third A1 rod pigment studied (paper II) was that of *R. catesbeiana*, which represents an opsin that functions together with the A2 chromophore in the tadpole stage and in parts of the adult retina that, moreover, vary with season (Reuter *et al.* 1971). It has a very much lower rate of thermal dark events than the toad rod pigments, although its absorbance spectrum is very similar (Donner *et al*. 1990). Yet, the temperature-induced λ_{max} shift measured by MSP was similar to that seen in the *Bufo* pigments, as was the qualitative observation that relative sensitivities in the far red increased with warming. The slopes were 1.55 x 10^{-5} m (warm) 1.64 x 10^{-5} m (cold) (ratio 1.058).

The photoactivation energies (E_a) of these three spectrally similar A1 rod pigments were calculated from the temperature effect on the long-wavelength limb of the spectra. Estimated E_a values were 44.3 ± 0.6 kcal/mol for *B. marinus* and 48.8 ± 0.5 kcal/mol for *B. bufo*. The two values are statistically different ($P < 0.001$). The *R. catesbeiana* estimate was E_a = 46.5 ± 0.8 kcal/mol, differing from both the former ($P < 0.05$).

One A1 photoreceptor with strongly differing absorbance spectrum was also studied (paper I): the "green" rod of *B. marinus*. However, the long-wavelength slopes could not be measured by ERG due to heavy contamination from a red-rod signal, and E_a could therefore not be determined. In MSP spectra, no visible effects of temperature changes were found: λ_{max} was 432.5 nm for cold spectra and 432.7 nm for warm spectra (see Table 1). MSP spectra did allow the further observation that, up to at least 530 nm, there was no temperature effect on long-wavelength sensitivities, implying that $E_a < 54$ kcal/mol.

4.1.2 COMPARISON OF PIGMENTS WITH A1 AND A2 CHROMOPHORE

In paper II, three pigments with A2 chromophore were studied by similar techniques as described above for A1 pigments: the A2 rod pigments of *R.*

catesbeiana and *Carassius carassius*, which are spectrally similar (λ_{max} = 525 and 526 nm, respectively), and the spectrally different L-cone pigment of *C. carassius* (λ_{max} = 619 nm). In all cases, the shift of λ_{max} due to temperature (ca. -0.6 nm per 10˚C) was similar to that in A1 (rhodopsin) rods (see above).

The warming-induced increase of relative long-wavelength sensitivities in the A2 pigments was smaller and started from longer wavelengths than in the A1 pigments. The long-wavelength slopes had the following values: *R. catesbeiana* A2 rod pigment 1.66 x 10^{-5} m (cold) and 1.57 x 10^{-5} m (warm); *C. carassius* A2 rod pigment 1.57 x 10^{-5} m (cold) and 1.45 x 10^{-5} m (warm); A2 L-cone pigment 1,99 x 10^{-5} m (cold) and 1.77 x 10^{-5} m (warm). The results are 70% - 89% of the theoretical Stiles predictions. The cold/warm slope ratio for the A2 pigments are: *R. catesbeiana* 1.054, *C. carassius* 1.079 (rods) and 1.123 (L-cones).

The E_a values estimated from these effects in *C. carassius* were 42.3 ± 0.6 kcal/mol (rods) and 38.3 ± 0.4 kcal/mol in L-cones, different at the $P < 0.01$ level and consistent with the inverse correlation of λ_{max} and E_a hypothesized in Fig. 8. Estimated E_a in *R. catesbeiana* A2 rod pigment was 44.2 ± 0.9 kcal/mol. This did not differ statistically significantly from the *C. carassius* rod pigment ($P = 0.13$), but was significantly smaller (one-sided $P < 0.05$) than the 46.5 ± 0.8 kcal/mol of the same opsin coupled to A1 chromophore.

The results are graphically summarized in Fig. 10 plotted as E_a against $1/\lambda_{max}$. The data points (red for A2 pigments, blue for A1), have been fitted with a least-squares regression line (weighted by their SEMs). The correlation ($r = 0.92$, $P < 0.01$) between λ_{max} and E_a evident in the Figure is difficult to interpret, however: firstly, the A1 pigments differ widely in E_a, although they have similar λ_{max}. Secondly, most of the correlation can be attributed to a difference between A1 and A2 pigments. Indeed, the only firm general conclusion appears to be that the chromophore shift from A1 to A2 is associated with a decrease in activation energy, correlating with the spectral red-shift.

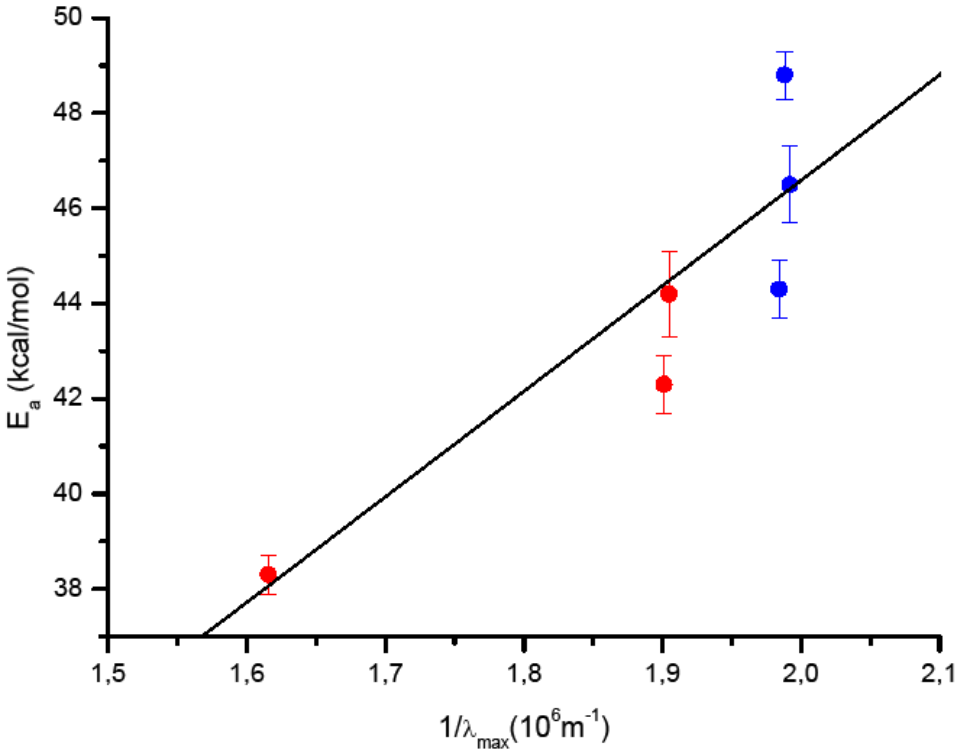


Fig. 10. The activation energies (E_a) of six visual pigments studied in papers I and II, plotted as functions of the (reciprocal) wavelength of maximum absorbance ($1/\lambda_{max}$). Red symbols denote A2 pigments (left to right: *Carassius carassius* L-cone and red rod, *Rana catesbeiana* red rod). Blue symbols denote A1 red rod pigments (from top to bottom: *Bufo bufo*, *Rana catesbeiana*, *Bufo marinus*). Error bars are SEMs, the straight line is a least-squares regression line fitted to the points weighted by their (reciprocal) SEM values (r^2 = 0.84).

4.2 VISUAL PIGMENTS IN RODS AND CONES OF STICKLEBACK POPULATIONS FROM DIFFERENT HABITATS: CHROMOPHORE USE AND PHOTIC ENVIRONMENT (*III*)

4.2.1 SPECTRAL CLASSES OF PHOTORECEPTORS: ONE ROD AND FOUR CONES

Absorbance spectra of rods and cones of eight populations of nine-spined sticklebacks (*Pungitius pungitius*) measured by MSP revealed one class of rods and four spectral classes of cones, arguably representing all four vertebrate cone opsin classes. The apparent absence of one or two of the cone classes in some of the populations was probably due only to incomplete sampling, and the tentative conclusion is that all populations have retained

the same basic complement of photoreceptors in different habitats. With the exception of two of the populations, they have been reproductively isolated since the ice shield receded after the last glaciation, some 8000 years ago (Donner 1995; Eronen 2001).

4.2.2 RODS: NO OPSIN DIFFERENCES BUT DIFFERENT CHROMOPHORE PROPORTIONS BETWEEN POPULATIONS

The rod opsin gene of each of the populations was sequenced from somatic DNA (three individuals from each). The nucleotide sequences indicated no differences in the derived amino acid sequence. Thus the spectral variation of rods between populations was attributed to different proportions of A1 and A2 chromophore and analyzed accordingly (see Materials and Methods). With respect to chromophore content in rods, the populations fell into two distinct groups: the A1-dominated Group 1 (λ_{max} 505-511 nm) and the A2-dominated Group 2 (λ_{max} 527-529 nm). As all animals were adults and had been kept in aquaria under standardized conditions with respect to light regime and temperature for at least half a year before measurements (in January-May), the differences are hardly due to “catching” the populations in somewhat randomly varying transitional stages (developmental or seasonal) of changing chromophore ratios, but are more constant properties. This was confirmed by studying three of the populations (two Group 1 and one Group 2) anew in the same season of another year (2011) after otherwise identical treatment. The rod chromophore proportions and the differences between two of these populations were remarkably constant over the years. In one population, however, the chromophore ratio changed systematically in the 2011 measurements (A1 → A2, decreasing A1 from 78% to 20% between March and September). This shows that *P. pungitius* as a species does have the capacity for physiological regulation of chromophore proportions.

When the measurements from different years in three of the eight populations were treated as independent data sets, the results comprised 11 data sets, summarized in Table 1: five Group 1 (data sets 1-5) and five Group 2 (data sets 6-10), plus the one referred to above with labile chromophore ratio (data set 11).

4.2.3 DIFFERENT CHROMOPHORE PROPORTIONS IN RODS AND CONES AND AMONG DIFFERENT CONE TYPES

The cone opsins could not be sequenced at the time the studies were carried out. Even given chromophore variation, however, it was possible to assign all cones to one of four distinct spectral classes based on λ_{max}: SWS1 407-412 nm, SWS2 426-458 nm, MWS 519-544 nm, and LWS 550-606 nm. The λ_{max} range within each class can be accounted for by chromophore differences

(Whitmore and Bowmaker 1989; Hárosi 1994) when allowance is made for the noisiness and small sample size of especially SWS cones. The further quantitative analysis of cones was thus based on the assumption that, just as in rods, spectral variation was due to varying chromophore proportions. The lower quality of cone MSP data in general, due to the small size of outer segments, rareness of some cone types, and bleaching problems in the earlier phases of the study, forced us to concentrate on MWS cones alone for the main analysis of chromophore content in cones. The results for all 11 data sets are summarized together with the rod results in Table 2.

In all Group 2 rod populations (data sets 6-10), the M-cone spectra were also shifted towards longer wavelengths and indicated a high proportion of A2 chromophore. However, even in the Group 1 populations (data sets 1-5), M-cone spectra indicated fairly high proportions of A2, and the difference compared with the A1-dominated rods was statistically significant in all ($P < 0.05$). In two data sets (#4 and 8 in Table 2) where the L-cone data was of sufficient quality to allow reliable comparison with M-cones, it was further shown that L- and M-cones could have significantly different chromophore proportions. In the Group 1 data set #4 with virtually pure A1 in the rods, the chromophore estimate for L-cones was also 100% A1, whereas that for the M-cones was 40 % A1 ($P < 0.001$) In the Group 2 data set #8, it was estimated that L-cones (like rods) had practically 0 % A1 chromophore, whereas M-cones had 23 % A1. About SWS cones, nothing more precise can be said than that the A1/A2 ratios seemed to vary over the full range 0-100 % A1. (See paper *III* for details.)

Table 2 Wavelengths of peak absorbance and chromophore content in rods and M-cones in eight populations of nine-spined sticklebacks as analyzed by three methods. (1) Running number used to identify the data set in the main Text. (2) Population, number of individuals (N) included, and the recording months and year. The abbreviation of the population is shown in capitals beside the name of the habitat. (3) Photoreceptor type and total numbers of cells from which spectra are included (n). Within each population, the top row(s) refers to rod values, the bottom row(s) (shaded) to M-cone values from the same sample of individuals. (4) Template λ_{max} ± SEM. Mean and standard error of the Govardovskii *et al.* (2000) parameter λ_{max} obtained by fitting sums of A1 and A2 templates to the full spectrum of each individual. (5) A1 (%): the percentage of A1 in the sum of A1 and A2 templates that provided the fit in column (4) (Method 1). (6) Parabola λ_{maxp} ± SEM. Mean and standard error of the wavelengths of peak absorbance obtained by least-squares fitting of second-order polynomials to data ± 30 nm and ± 50 nm around the provisional peaks obtained by template-fitting to each individual. (7) A1 (%): the percentage of A1 in a mixture of an A1-A2 pigment pair that would give the λ_{maxp} shown in column (6). For rods, the value has been calculated under the assumption that the pure A1 component peaks at 504.8 nm (first number) and at 503.3 nm (number in parenthesis). For cones, the pure A1 pigment has been assumed to peak at 512.5 nm (Method 2). (8-9) Results of principal component analysis (PCA). Peak wavelengths of major PCA components, and percent of the total variance explained by each of them.

Number	Population and recording season (N)	Photoreceptor type (n)	Template λ_{max} ± SEM	A1 (%)	Parabola λ_{max} ± SEM	A1 (%)	PCA component	Percent explained
1	Abbortjärn / ABB (6)	Rod (107)	507,8 ± 0,6	96,9	504,0 ± 0,6	98,3 (91,1)	501,8	38,3
	2/2008	Cone (126)	519,2 ± 0,7	68,9	517,8 ± 0,6	72,3	516,8	58,1
2	Iso-porontima / ISO (5)	Rod (137)	511,0 ± 1,4	91,6	507,9 ± 0,9	77,7 (70,3)	503,5	57,0
	2/2008						571,4	2,7
		Cone (75)	523,3 ± 1,7	67,7	522,4 ± 1,3	53,3	515,8	59,1
							580,6	2,4
3	Pyöreälampi / PYO (6)	Rod (108)	504,8 ± 0,4	85,1	503,0 ± 0,3	100,0 (98,3)	504,5	60,0
	4/2008	Cone (59)	518,6 ± 0,6	71,8	519,0 ± 0,6	65,0	526,2	60,0
							515,2	7,6
4	Pyöreälampi / PYO (7/6)	Rod (225)	507,9 ± 0,6	100,0	505,9 ± 0,5	92,4 (83,1)	507,2	65,9
	2-5/2011						574,2	1,0
		Cone (131)	534,3 ± 1,8	49,0	531,6 ± 1,6	30,3	532,5	77,0
							519,8	7,1
							546,8	4,9
							589,5	2,6
5	Rytilampi / RYT (6)	Rod (180)	507,9 ± 1,5	88,3	505,5 ± 1,0	92,0 (86,2)	500,8	60,0
	2-3/2008						560,8	2,3
		Cone (108)	520,3 ± 1,0	84,0	520,2 ± 0,9	62,2	517,4	66,9
6	Bölesviken BOL (7/6)	Rod (153)	527,6 ± 1,3	27,6	527,5 ± 0,9	11,6 (6,6)	523,0	41,3
	2/2008	Cone (92)	541,7 ± 1,6	0,0	540,1 ± 1,1	8,2	535,5	45,6
7	Helsinki / HEL (5)	Rod (98)	527,3 ± 1,4	0,0	527,1 ± 1,6	10,8 (5,3)	-	-
	3/2008	Cone (78)	538,0 ± 2,7	55,5	536,3 ± 1,1	15,5	-	-
8	Helsinki / HEL (8/6)	Rod (251)	529,0 ± 0,6	1,9	524,7 ± 0,7	18,8 (12,7)	525,2	79,0
	1-5/2011	Cone (242)	544,8 ± 2,3	37,7	543,3 ± 2,4	8,6	530,5	66,9
							618,5	1,1
9	White Sea / WHI (5/3)	Rod (112)	528,6 ± 1,4	82,5	525,5 ± 0,8	19,1 (10,8)	536,2	54,2
	4-10/2008						569,9	5,6
		Cone (31)	536,0 ± 1,1	7,7	534,1 ± 2,3	17,1	523,8	35,1
							536,3	16,3
10	Mashinnoje /MAS (6)	Rod (145)	529,5 ± 0,6	6,6	528,9 ± 0,6	7,9 (3,0)	525,4	45,6
	8-9/2008	Cone (95)	543,5 ± 1,0	2,1	542,5 ± 1,5	4,1	535,1	34,3
11	Rytilampi / RYT (6)	Rod (148)	516,5 ± 3,2	60,9	514,0 ± 2,4	64,5 (56,2)	513,5	66,8
	3-5/2011						569,2	2,1
		Cone (212)	540,6 ± 1,6	60,9	537,9 ± 1,2	11,5	534,2	61,6
							603,5	2,1

4.2.4 SPECTRA OF THE DOWNWELLING LIGHT IN THE HABITAT OF THE POPULATIONS STUDIED

The spectra of downwelling light were measured in September 2011 in daylight at different depths in four of the eight habitats of the populations studied (Fig. 11). The peak values of transmittance were Helsinki (~570 nm), Iso-Porontima (~575 nm), Pyöreälampi (~565 nm), and Rytilampi (~565 nm). All the measured habitats were rather shallow, as the deepest measured lake, Iso-Porontima, was 15 m deep and Rytilampi pond was only 2 m deep. The other habitats seemed, by visual inspection, to be very similar to those measured, with the exception of Abbortjärn in Sweden, which was a typical humic pond with strongly red-shifted transmittance.

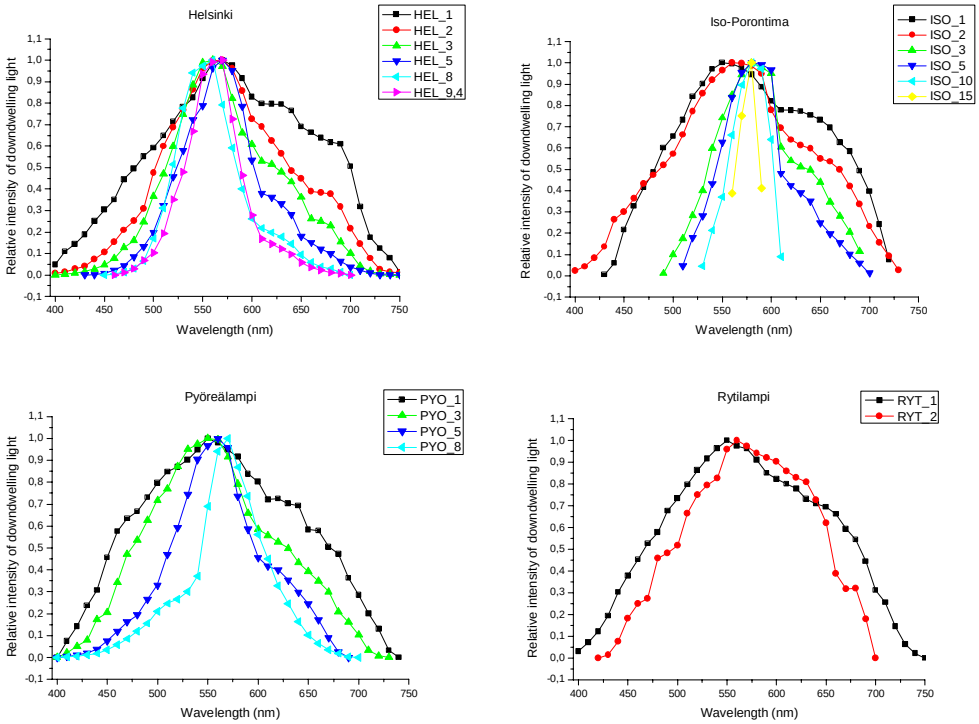


Fig. 11. Relative intensity of downwelling light (normalized to unity at peak) of four of the habitats of nine-spined sticklebacks studied. In insets measured depths in meters.

5 DISCUSSION

5.1 THE RELATION BETWEEN λ_{MAX}, E_A, AND THERMAL ACTIVATION RATES

The primary purpose of Part 1 of this thesis (papers I and II) was to test the scheme illustrated in Fig. 8, often loosely referred to as the "Barlow hypothesis" (cf. Barlow 1957). At the time, a general correlation between λ_{max} and dark noise had been described in the sample of photoreceptors where both had been measured: in rods for rates of thermal dark events (Donner *et al*. 1990; Firsov and Govardovskii 1990; cf. Fyhrquist 1999) and in cones for the power of dark noise in the frequency band of photoresponses (Rieke and Baylor 2000). On the other hand, significant deviations from the hypothesized strict interrelation between E_a, λ_{max}, and thermal activations were known. The rate of thermal dark events in toad "green" rods appeared way too high (Matthews 1984) and in the activation energies of 5 amphibian pigments studied by Koskelainen *et al.* (2000), the only clear correlation found was that pigments with A2 chromophore had lower E_a than those with A1 chromophore.

The results of papers I and II as summarized in Fig. 10 support the conclusion of Koskelainen *et al*. (2000) that the A1 → A2 switch is associated with lowered E_a. Especially significant is the difference here found between *Rana catesbeiana* A1 and A2 versions of the same opsin, which can in turn be directly correlated with the much higher rate of thermal dark events in the A2 rods compared with the A1 rods of the same species (Donner *et al.* 1990). On the other hand, the three A2 pigments may suggest a chromophore-independent correlation, although it has to be noted that pigment with the lowest E_a is a cone pigment, whilst the two rod pigments that both have similar λ_{max} again have rather different E_a. The three A1 rod opsins with almost the same λ_{max} argue strongly against the idea of a very strict λ_{max}-E_a coupling.

Ala-Laurila *et al.* (2004a) assembled a larger sample of 12 λ_{max}-E_a determinations from different sources, including the present ones, and concluded that there is indeed a loose correlation independent of chromophore differences, but that the only strict rule is the E_a decrease associated with the A1 → A2 switch. The authors suggested that there may be some freedom for independent tuning of λ_{max} and E_a due to the multiple ways (different amino acid sequences) by which virtually indistinguishable absorbance spectra (i.e., with the same λ_{max}) can be produced. One may speculate that natural selection will "try to keep" E_a as high as possible while tuning pigments for high long-wavelength sensitivity. This was the idea that Fyhrquist *et al.* (1998b) had pursued when trying to identify amino acid substitutions specifically associated with thermal stability in the spectrally

similar rhodopsins of *Bufo marinus, Bufo bufo, and Rana catesbeiana*. However, the differences in opsin sequences were too many to allow more than a tentative assignment of some "candidate" residues.

Of course, the important variable from the viewpoint of visual function is not E_a as such, but the rate of thermal pigment activations (noise). The relevance of E_a to thermal noise was completely unclear at the time of studies I and II. The temperature-dependence of the rate of thermal events as analyzed by Baylor *et al.* (1980) had indicated activation energy of only ca. 22 kcal/mol for the thermal process, quite incompatible with the 40-50 kcal/mol characteristic of photoactivation (Lythgoe and Quilliam 1938; St. George 1952; Cooper 1979). Therefore, it was thought that the molecular routes of photoactivation and thermal activation are so different that E_a might be practically unrelated to dark event rates (e.g., Barlow *et al.* 1993). However, a hypothesis published a year after paper II (Ala-Laurila *et al.* 2004b) suggested that the difference might be just an analytical artefact. If the temperature dependence of dark event rates is analyzed not based on Boltzmann statistics, as Baylor *et al.* (1980) did, but observing that in a complex molecule like retinal, thermal activation depends on the (Hinshelwood) statistics of multiple vibrational modes, the thermal activation energy converges with E_a for quite realistic numbers of vibrational modes.

On this general basis, Luo *et al.* (2011) have analyzed a new set of experimental data of the highest quality, recorded from single cells by the suction-pipette technique. They suggest a very strict connection between E_a, λ_{max}, and thermal activation rates. They got a value of ca. 48 kcal/mol for the activation energy of *B. marinus* rod pigment, significantly different from the present value (44.3 kcal/mol) for the same pigment and broadly consistent with the *B. bufo* value obtained here (48.8 kcal/mol). This reminds us that the ERG (as potentially any other technique, including the suction pipette technique) is sensitive to significant systematic error and unexplained variability. Yet it would be premature to explain all deviations observed here (Fig. 8) and elsewhere just by artefacts of the ERG technique. Moreover, the spectrally similar A1 rods of *R. catesbeiana* and *B. marinus* undoubtedly have very different rates of discrete dark events (Baylor *et al.* 1980; Donner *et al.* 1990). Comparing *Rana* and *Bufo* opsins, Fyhrquist *et al.* (1998a) found 16 non-conserved amino acid substitutions and 6 sites involving the loss or gain of a hydroxyl group. Although they could not pin down specific residues affecting thermal stability, the different amino acid sequences that underlie similar absorbance spectra but different rates of thermal activation levels of visual pigments with the same chromophore (A1) suggest that the opsin can regulate in what way different thermal vibration modes contribute towards isomerisation of the chromophore and activation of the pigment. At this stage, it seems best to think that the question how "strict" the correlations shown in Fig. 8 are, has not been finally settled. It may also be added that there have been other hypotheses than the statistical one of Ala-

Laurila *et al.* (2004b) attempting to explain the mechanisms of photoactivation and thermal activation (e.g., Bókkon *et al.* 2009, Khrenova *et al.* 2009, Lórenz-Fonfriá *et al.* 2010).

Be that as it may, one uncontested conclusion is that A2 pigments have lower photoactivation energies than A1 pigments based on the same opsin, and this seems to be associated with higher thermal noise. Ala-Laurila *et al*. (2007) did chromophore substitution experiments in salamander rods, measuring dark event rates as function of A2 content. They showed that the rate rose linearly with the amount of A2 in the rod, and that the difference for pure A2 compared with pure A1 was at least 36-fold. Kefalov *et al.* (2005) showed the same effect on larval salamander red cones as they found that 11–*cis* A1 red cone pigment has a rate of thermal activation eleven–fold lower than the corresponding rate for 11–*cis* A2 cone pigment. This clear difference between A1 and A2 pigments forms the background for the following discussion of Part 2 of the present thesis.

5.2 CHROMOPHORE USE IN RODS AND CONES OF NINE-SPINED STICKLEBACKS

The studies on *Pungitius pungitius* deal with the functional significance of the different molecular properties of the A1 and A2 pigments that were considered in Part 1, in the context of population ecology. The basic finding was that the differences between the isolated populations were confined to chromophore differences. All spectral variation within each of the five basic spectral classes of photoreceptors could be explained by variation in the chromophore proportions, without assuming any differences in the respective opsins. This gives a firm basis for functional predictions, as the correlation of λ_{max} and dark noise appears reasonably tight for chromophore-determined variation. As the (present) light environments are known, relative quantum catch (QC) and conceptual signal-to-noise ratios (SNRs) achieved by the A1 and A2 versions of the different pigments can be estimated with some confidence. In paper III, the estimations were done graphically utilizing “template” functions calculated and published by Jokela-Määttä *et al.* (2007). The functions give relative QC and SNR as functions of λ_{max} for A1 and A2 pigments in a number of model light environments. Admittedly, the SNR calculations are based on the *average* relation between λ_{max} and thermal event rates as modelled by Ala-Laurila *et al.* (2007), which may be too conservative an assumption for the noise difference between A2 and A1 pigments.

Most of the light environments in paper III were close to or intermediate between Jokela-Määttä’s model environments B (open Baltic Sea) and Bp (Pojoviken Bay of the Baltic Sea). Fig. 12 shows the curves giving (relative) QC and SNR as functions of λ_{max} in these two environments.

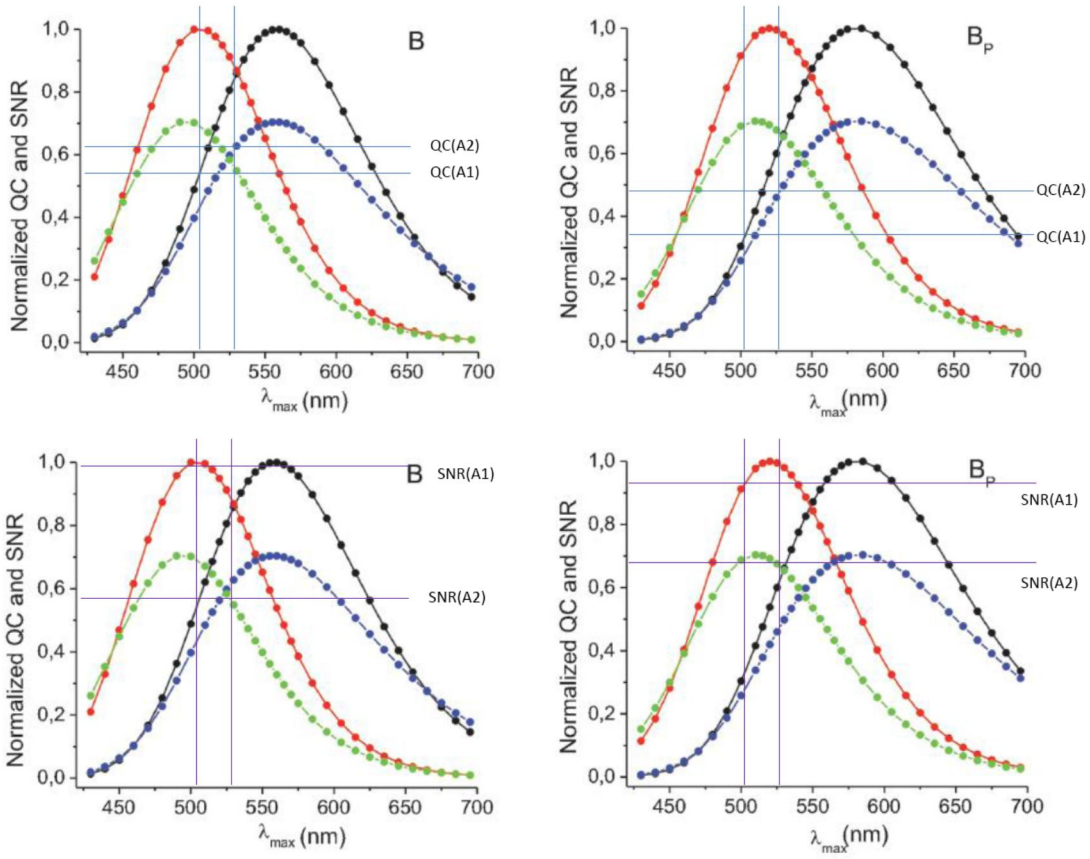


Fig. 12. Theoretical performance measures of visual pigments in two spectral environments (for B peaking at 560 nm in the left-hand panels and B_P peaking at 580 nm in the right-hand panels) plotted as functions of λ_{max} of the pigments. Red and black curves refer to A1 pigments, green and blue curves to A2 pigments. In each panel, the two curves that peak at shorter wavelengths (red and green) give the signal-to-noise ratio when pigment noise is dominant (i.e., at the absolute visual threshold), and the two curves that peak at longer wavelengths (black and blue) give quantum catch. All values are given as fractions of the maximum values for A1 pigments, which have been normalized to one. The A2 curves have lower peaks due to the lower photosensitivity of A2 pigments (Dartnall 1972). The top and bottom panels reproduce exactly the same sets of curves; the reason for duplicating them is to avoid clutter of the straight lines exemplifying how QC and SNR are read. *Top panels*: to get QC for rod A1 pigment, take the value of the black curve at 504 nm, for the A2 version, take the value of the blue curve at 531 nm. *Bottom panels*: to get SNR for rod A1 pigment, take the value of the red curve at 504 nm, for the A2 version, take the value of the green curve at 531 nm. With permission from Jokela-Määttä *et. al.* 2007.

5.2.1 RODS

The straight lines in Fig. 12 illustrate how QC and SNR are read from the curves for the A1 and A2 versions of the stickleback rod pigment (λ_{max} = 504 nm and 531 nm, respectively). The conclusion is that even in the relatively short-wavelength model habitat with light peaking at 560 nm (left-hand panels), the A2 pigment would catch more quanta, and the difference

becomes even greater for environments with light spectra shifted towards longer wavelength (right-hand panels for light peaking at 580 nm). By contrast, the A1 pigment gives higher SNR in all environments.

Yet, rods used predominantly A1 in some populations and predominantly A2 in others, and there was no clear pattern to this. In fact, the only easy generalization was that freshwater populations had A1-dominated rods and brackish-water ("sea") populations A2-dominated rods. This is contrary to the common generalization that freshwater fish tend to have more A2-dominated pigments because the spectral transmission is red-shifted (Jerlov 1976), whereas marine and brackish-water fishes tend to be A1-dominated (Wald 1937, 1939; Bridges 1972; Jokela-Määttä *et al*. 2007). On the other hand, the coastal brackish-water environments of the sticklebacks are long-wave-shifted compared with real ocean water.

The results do not allow any simple interpretation, and while the analysis helps to structure the problem, it gives no clear answers. It is important to bear in mind that the SNR discussed above refers to absolute visual threshold, when intrinsic "dark" noise is the main limiting factor. As soon as there is a little more light and photon fluctuations become dominant, the SNR of vision will increase monotonically with increasing QC. Thus the relative functional usefulness of A1 (higher "dark" SNR) and A2 (higher QC) depends on the absolute level of illumination where visual behaviour is critically limited by rod vision. This in turn may vary e.g. with season, depth of the water body, or lifestyle of the fish.

Indeed, the capability of changing chromophore proportions with season was evident in one data set (#11 in Table 2). It is quite likely that the other populations have the same capability but we did not observe it, as we were especially keeping the aquarium conditions as well as the recording season standardized and constant. The stable contrasts between populations described here do demonstrate genetically determined differences in the systems regulating the A1/A2 balance (maybe in the "norms of reaction" to different environmental cues) but they do not demonstrate, for example, that rods of Group 1 populations are A1-dominated under all conditions.

5.2.2 CONES

The most interesting discovery was that in populations with A1 dominated rods (Group 1), the fraction of A2 chromophore in the M-cones was significantly higher than in the rods. Differential chromophore use of rods and cones has not, to our knowledge, been reported before. Further, in two cases L-cone recordings were extensive enough to allow the conclusion that L-cones differed from M-cones in chromophore use. These observations are intriguing from the viewpoint of both function and mechanism. The former is exemplified by calculations in paper III showing that selective A1/A2 targeting to rods and cones, and to different types of cones, may be used in a

sophisticated manner to achieve tuning for high sensitivity of single photoreceptors (as considered above) and for good difference signals of cone pairs (wavelength discrimination) in varying light environments. It would be interesting to chart the full potential of chromophore-tuning of each cone type independently in its effects on the colour space of the fish. Some deep-sea fish are known to handle A1 and A2 selectively in rods to create cells with different spectral sensitivities based on a single opsin (Bowmaker *et al.* 1988; Crescitelli 1989).

In spite of limitations, mechanisms for chromophore delivery and uptake in different species are likely to be more versatile and specific than known at present. This field would certainly merit further study. The discovery of the cone-specific Müller-cell delivery pathway besides the “traditional” bulk delivery from the RPE was a beginning. However, cone/rod selectivity in mammals, having only one chromophore (A1), is of course related only to regeneration speed in the different kinds of photoreceptors. Selective chromophore handling at different points en route to the visual pigment has the greatest potential in species with the A1/A2 system. Theoretically, the complex task is to allocate optimal proportions of more red-sensitive, noisy A2 and more blue-sensitive, stable A1 chromophore in different photoreceptor types in view of different demands on photon catch and noise control in scotopic and photopic conditions.

6 CONCLUSIONS

1. Visual pigments using A2 chromophore (n = 3) were found to have lower activation energies (E_a) than those using A1 chromophore (n = 3). This is known to correlate with high thermal noise, which degrades visual sensitivity. On the basis of this small sample of pigments, however, it was not possible to decide whether there is a chromophore-independent inverse correlation between λ_{max} and E_a. The latter correlation has been firmly established in later studies by others.

2. Significant differences in A1-A2 chromophore use between rods and cones and between populations living in different light environments were found in the nine-spined stickleback. Functionally, rod-cone difference in chromophore use might serve high absolute sensitivity (by A1, low noise) in rod vision, and good wavelength discrimination (by A2, improving the spectral match to "yellowish" environments) in brighter light in cone vision. Mechanistically it indicates the presence of differential chromophore delivery pathways to rods and cones.

REFERENCES

Aguilar, M., & Stiles, W. S. (1954). Saturation of the rod mechanism of the retina at high levels of stimulation. Optica Acta, 1, 59-65.

Aho, A.-C. (1988). Low retinal noise in animals with low body temperature allows high visual sensitivity. Nature, 334, 348-350.

Ala-Laurila, P., Donner, K., Crouch, R. K., & Cornwall, M. C. (2007). Chromophore switch from 11-cis-dehydroretinal (A2) to 11-cis-retinal (A1) decreases dark noise in salamander red rods. The Journal of Physiology, 585, 57-74.

Ala-Laurila, P., Donner, K., & Koskelainen, A. (2004a). Thermal activation and photoactivation of visual pigments. Biophysical Journal, 86, 3653-3662.

Ala-Laurila, P., Kolesnikov, A. V., Crouch, R. K., Tsina, E., Shukolyukov, S. A., Govardovskii, V. I., Koutalos, Y., Wiggert, B., Estevez, M. E., & Cornwall, M. C. (2006). Visual cycle: Dependence of retinol production and removal on photoproduct decay and cell morphology. The Journal of General Physiology, 128, 153-169.

Ala-Laurila, P., Pahlberg, J., Koskelainen, A., & Donner, K. (2004b). On the relation between the photoactivation energy and the absorbance spectrum of visual pigments. Vision Research, 44, 2153-2158.

Ala-Laurila, P., Saarinen, P., Albert, R., Koskelainen, A., & Donner, K. (2002). Temperature effects on spectral properties of red and green rods in toad retina. Visual Neuroscience, 19, 781-792.

Allen, D. M., & McFarland, W. N. (1973). The effect of temperature on rhodopsin-porphyropsin ratios in a fish. Vision Research, 13, 1303-1309.

Allen, D. M., McFarland, W. N., Munz, F. W., & Poston, H. A. (1973). Changes in the visual pigments of trout. Canadian Journal of Zoology, 51, 901-914.

Alpern, M., Fulton, A. B., & Baker, B. N. (1987). "Self-screening" of rhodopsin in rod outer segments. Vision Research, 27, 1459-1470.

Altenbach, C., Yang, K., Farrens, D. L., Farahbakhsh, Z. T., Khorana, H. G., & Hubbell, W. L. (1996). Structural features and light-dependent changes in the cytoplasmic interhelical EF loop region of rhodopsin: A site-directed spin-labeling study. Biochemistry, 35, 12470-12478.

Archer, S. N. (1999). Light and photoreception: Visual pigments and photoreception. In: Archer, S. N., Djamgoz, E. R., Partridge, J. C., & Vallerga, S. (Eds.), Adaptive mechanisms in the ecology of vision, Dordrecht: Kluwer Academic Publishers, 25-41.

Autrum, H. (1943). Über kleinste Reize bei Sinnesorganen. Biologisches Zentralblatt, 63, 209–236.

Bailes, H. (2007). Visual pigments in a living fossil, the Australian lungfish *Neoceratodus forsteri*. BMC Evolutionary Biology, 7, 200, doi: 10.1186/1471-2148-7-200

Barlow, H. B. (1956). Retinal noise and absolute threshold. Journal of the Optical Society of America, 46, 634-639.

Barlow, H. B. (1957). Purkinje shift and retinal noise. Nature, 179, 255-256.

Barlow, R. B., Birge, R. R., Kaplan, E., & Tallent, J. R. (1993). On the molecular origin of photoreceptor noise. Nature, 366, 64-66.

Baylor, D. A., Lamb, T. D., & Yau, K. W. (1979). Responses of retinal rods to single photons. The Journal of Physiology, 288, 613-634.

Baylor, D. A., Matthews, G., & Yau, K. W. (1980). Two components of electrical dark noise in toad retinal rod outer segments. Journal of Physiology, 309, 591-621.

Baylor , D. A., Nunn, B. J., & Schnapf, J. L. (1984). The photocurrent, noise and spectral sensitivity of rods of the monkey *Macaca fascicularis*. Journal of Physiology, 357, 575-607.

Beatty, D. D. (1969). Visual pigment changes in juvenile kokanee salmon in response to thyroid hormones. Vision Research, 9, 855-864.

Beatty, D. D. (1984). Visual pigments and the labile scotopic visual system of fish. Vision Research, 24, 1563-1573.

Bókkon, I., & Vimal, R. L. P. (2009). Retinal phosphenes and discrete dark noises in rods: A new biophysical framework. Journal of Photochemistry and Photobiology B: Biology. 96, 255-259.

Bowmaker, J. K. (1991). The evolution of vertebrate visual pigments and photoreceptors. In: Cronly-Dillon, J. R., & Gregory, R. L. (Eds.), Vision and visual dysfunction, vol. 2: Evolution of the eye and visual system. Boca Raton: CRC Press, 63-81.

Bowmaker, J. K. (1995). The visual pigments of fish. Progress in Retinal and Eye Research, 15, 1-31.

Bowmaker, J. K., Dartnall, H. J. A., & Herring, P. J. (1988). Longwave-sensitive visual pigments in some deep-sea fishes: Segregation of 'paired' rhodopsins and porphyropsins. Journal of Comparative Physiology A, 163, 685-698.

Bownds, D. (1967). Site of attachment of retinal in rhodopsin. Nature, 216, 1178-1181.

Bridges, C. D. (1965). Visual pigments in a fish exposed to different light-environments. Nature, 206, 1161-1162.

Bridges, C. D. (1967). Spectroscopic properties of porphyropsins. Vision Research, 7, 349-369.

Bridges, C. D. B. (1972). The rhodopsin-porphyropsin visual system. In: Dartnall, H. J. A. (Ed.), Handbook of sensory physiology VII/1: Photochemistry of vision. Berlin-Heidelberg-New York: Springer-Verlag, 417-480.

Britt, L. L., Loew, E. R., & McFarland, W. N. (2001). Visual pigments in the early life stages of Pacific Northwest marine fishes. The Journal of Experimental Biology, 204, 2581-2587.

Bunt, A. H., & Saari, J. C. (1982). Fucosylated protein of retinal cone photoreceptor outer segments: Morphological and biochemical analyses. The Journal of Cell Biology, 92, 269-276.

Bunt-Milam, A. H., & Saari, J. C. (1983). Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. The Journal of Cell Biology, 97, 703-712.

Campbell, F. W., & Rushton, W. A. (1955). Measurement of the scotopic pigment in the living human eye. The Journal of Physiology, 130, 131-147.

Carleton, K. L., & Kocher, T. D. (2001). Cone opsin genes of African cichlid fishes: Tuning spectral sensitivity by differential gene expression. Molecular Biology and Evolution, 18, 1540-1550.

Clark, J. A., Cena, K., & Monteith, J. L. (1973). Measurements of the local heat balance of animal coats and human clothing. Journal of Applied Physiology, 35, 751-754.

Clarke, G. L., & James, H. J. (1939). Laboratory analysis of the selective absorption of light by sea water. Journal of the Optical Society of America, 29, 43-53.

Cooper, A. (1979). Energy uptake in the first step of visual excitation. Nature, 282, 531-533.

Crescitelli, F. (1972). The visual cells and visual pigments of the vertebrate eye. In: Dartnall, H. J. A. (Ed.), Photochemistry of vision: Handbook of sensory physiology. Berlin-Heidelberg-New York: Springer-Verlag, 245-263.

Crescitelli, F. (1973). The visual pigment system of *Xenopus laevis*: Tadpoles and adults. Vision Research, 13, 855-865.

Crescitelli, F. (1989). The visual pigments of a deep-water malacosteid fish. Journal of the Marine Biological Association of the United Kingdom, 69, 43-51.

Crouch, R. K., Hazard, E. S., Lind, T., Wiggert, B., Chader, G., & Corson, D. W. (1992). Interphotoreceptor retinoid-binding protein and alpha-tocopherol preserve the isomeric and oxidation state of retinol. Photochemistry and Photobiology, 56, 251-255.

Cuthill, I. C., Bennett, A. T. D., Partridge, J. C., & Maier, E. J. (1999). Plumage reflectance and the objective assessment of avian sexual dichromatism. The American Naturalist, 153, 183-200.

Dartnall, H. J. A. (1953). The interpretation of spectral sensitivity curves. British Medical Bulletin, 9, 24-30.

Dartnall, H. J. A. (1972). Photosensitivity. In: Dartnall, H. J. A. (Ed.), Photochemistry of vision: Handbook of sensory physiology. Berlin-Heidelberg-New York: Springer-Verlag, 122-145.

Dartnall, H. J. A., & Goodeve, C. F. (1937). Scotopic luminosity curve and the absorption spectrum of visual purple. Nature, 139, 409-410.

Dartnall, H. J. A., Lander, M. R., & Munz, F. W. (1961). Periodic changes in the visual pigment of a fish. In: Christensen, B., & Buchmann, B. (Eds.), Progress in photobiology. Amsterdam: Elsevier, 203–213.

Dartnall, H. J. A., & Lythgoe, J. N. (1965). The spectral clustering of visual pigments. Vision Research, 5, 81-100.

de Vries, H. (1948). Der Einfluss der Temperatur des Auges auf die spektrale Empfindlichkeitskurve. Experientia, 4, 357.

Dominy, N. J., & Lucas, P. W. (2001). Ecological importance of trichromatic vision to primates. Nature 410, 363-366.

Donner, J., (1995). The Quaternary history of Scandinavia. Cambridge: Cambridge University Press.

Donner, K., (1992). Noise and the absolute thresholds of cone and rod vision. Vision Research, 32, 853-866.

Donner, K., (1999), Begränsningar och biologiska val i synsystemet. Sphinx, Societas Scientiarum Fennica Yearbook, 1998-1999, 17-28.

Donner, K., Firsov, M. L., & Govardovskii, V. I. (1990). The frequency of isomerization-like 'dark' events in rhodopsin and porphyropsin rods of the bull-frog retina. The Journal of Physiology, 428, 673-692.

Donner, K., Hemilä, S., & Koskelainen, A. (1998). Light adaptation of cone photoresponses studied at the photoreceptor and ganglion cell levels in the frog retina. Vision Research, 38, 19-36.

Douglas, R. H., & Partridge, J. C. (1997). On the visual pigments of deep-sea fish. Journal of Fish Biology, 50, 68-85.

Duan, W., & Fuerst, P. A. (2001). Isolation of a sex-linked DNA sequence in cranes. The Journal of Heredity, 92, 392-397.

Eilers, M., Reeves, P.J., Ying, W., Khorana, H.G., & Smith, S. O. (1999). Magic angle spinning NMR of the protonated retinylidene schiff base nitrogen in rhodopsin: Expression of 15N-lysine- and 13C-glycine-labeled opsin in a stable cell line. Proceedings of the National Academy of Sciences of the United States of America, 96, 487-492.

Eisenfeld, A. J., Bunt-Milam, A. H., & Saari, J. C. (1985). Immunocytochemical localization of interphotoreceptor retinoid-binding protein in developing normal and RCS rat retinas. Investigative Ophthalmology & Visual Science, 26, 775-778.

Eronen, M. (2001). Rates of Holocene isostatic uplift and relative sea-level lowering of the Baltic in SW Finland based on studies of isolation contacts. Boreas, 30, 17-30.

Fain, G. L., Matthews, H. R., & Cornwall, M. C. (1996). Dark adaptation in vertebrate photoreceptors. Trends in Neurosciences, 19, 502-507.

Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., & Khorana, H. G. (1996). Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. Science, 274, 768-770.

Fernald, R. D. (2006). Casting a genetic light on the evolution of eyes. Science, 313, 1914-1918.

Field, G. D., Sampath, A. P., & Rieke, F. (2005). Retinal processing near absolute threshold: From behavior to mechanism. Annual Review of Physiology, 67, 491-514.

Firsov, M. L., Donner, K., & Govardovskii, V. I. (2002). pH and rate of 'dark' events in toad retinal rods: Test of a hypothesis on the molecular origin of photoreceptor noise. The Journal of Physiology, 539, 837-846.

Fuller, R. C., & Montgomery, W. L. (2003). Disentangling female mate choice and male competition in the rainbow darter, *Etheostoma caeruleum*. Copeia, 2003, 138-148.

Fyhrquist, N. (1999). Spectral and thermal properties of amphibian visual pigments related to molecular structure. PhD-thesis, University of Helsinki.

Fyhrquist, N., Donner, K., Hargrave, P., McDowell, J. H., Popp, M. P., & Smith, W. C. (1998a). Rhodopsins from three frog and toad species: Sequences and functional comparisons. Experimental Eye Research, 66, 295-305.

Fyhrquist, N., Donner, K., Hargrave, P. A., McDowell, J. H., Popp, M. P., & Smith, W. C. (1998b). Rhodopsins from three frog and toad species: Sequences and functional comparisons. Experimental Eye Research, 66, 295-305.

Goldsmith, T. (1990). Optimization, constraint, and history in the evolution of eyes. The Quarterly Review of Biology, 65, 281-322.

Govardovskii, V. I., Fyhrquist, N., Reuter, T., Kuzmin, D. G., & Donner, K. (2000). In search of the visual pigment template. Visual Neuroscience, 17, 509-528.

Govardovskii, V. I., & Zueva, L. V. (1988). A simple highly sensitive recording microspectrophotometer. Tsitologiia, 30, 499-502.

Hargrave, P. A., McDowell, J. H., Curtis, D. R., Wang, J. K., Juszczak, E., Fong, S., Mohana Rao, J. K., & Argos, P. (1983). The structure of bovine rhodopsin. European Biophysics Journal, 9, 235-244.

Hargrave, P. A., McDowell, J. H., Feldmann, R. J., Atkinson, P. H., Mohana Rao, J. K., & Argos, P. (1984). Rhodopsin's protein and carbohydrate structure: Selected aspects. Vision Research, 24, 1487-1499.

Hárosi, F. I. (1975). Absorption spectra and linear dichroism of some amphibian photoreceptors. The Journal of General Physiology, 66, 357-382.

Hárosi, F. I. (1994). An analysis of two spectral properties of vertebrate visual pigments. Vision Research, 34, 1359-1367.

Hecht, S., Haig, C., & Chase, A. M. (1937). The influence of light adaptation on subsequent dark adaptation of the eye. The Journal of General Physiology, 20, 831-850.

Heikkinen, H., Nymark, S., Donner, K., & Koskelainen, A. (2009). Temperature dependence of dark-adapted sensitivity and light-adaptation in photoreceptors with A1 visual pigments: A comparison of frog L-cones and rods. Vision Research, 49, 1717-1728.

Hubbard, R., & Wald, G. (1952). Cis-trans isomers of vitamin A and retinene in the rhodopsin system. The Journal of General Physiology, 36, 269-315.

Hunt, D. M., Dulai, K. S., Partridge, J. C., Cottrill, P., & Bowmaker, J. K. (2001). The molecular basis for spectral tuning of rod visual pigments in deep-sea fish. The Journal of Experimental Biology, 204, 3333-3344.

Jacobs, G. H. (1981). Comparative color vision. New York: Academic Press.

Jacobs, G. H. (1983). Within-species variations in visual capacity among squirrel monkeys (*Saimiri sciureus*): Sensitivity differences. Vision Research, 23, 239-248.

Jacobs, G. H. (2008). Primate color vision: a comparative perspective. Visual Neuroscience 25, 619-633.

Jerlov, N. G. (1976). Marine optics. Amsterdam: Elsevier.

Jin, M., Li, S., Nusinowitz, S., Lloyd, M., Hu, J., Radu, R. A., Bok, D., & Travis, G. H. (2009). The role of interphotoreceptor retinoid-binding protein on the translocation of visual retinoids and function of cone photoreceptors. The Journal of Neuroscience, 29, 1486-1495.

Jokela-Määttä, M., Smura, T., Aaltonen, A., Ala-Laurila, P., & Donner, K. (2007). Visual pigments of Baltic sea fishes of marine and limnic origin. Visual Neuroscience, 24, 389-398.

Jokela-Määttä, M., Vartio, A., Paulin, L., & Donner, K. (2009). Individual variation in rod absorbance spectra correlated with opsin gene polymorphism in sand goby (*Pomatoschistus minutus*). The Journal of Experimental Biology, 212, 3415-3421.

Jones, G. J., Crouch, R. K., Wiggert, B., Cornwall, M. C., & Chader, G. J. (1989). Retinoid requirements for recovery of sensitivity after visual-pigment bleaching in isolated photoreceptors. Proceedings of the National Academy of Sciences of the United States of America, 86, 9606-9610.

Jurkowitz, L., Loeb, J. N., Brown, P. K., & Wald, G. (1959). Photochemical and stereochemical properties of carotenoids at low temperatures. Nature, 184, 614-624.

Kandori, H., Shichida, Y., & Yoshizawa, T. (2001). Photoisomerization in rhodopsin. Biochemistry, 66, 1197-1209.

Kefalov, V. J., Fu, Y., & Yau, K. W. (2005). Higher rate of thermal activation of red cone pigments with 11-cis A2 compared to 11-cis A1 retinal as chromophore. ARVO Meeting Abstracts, 46, 2264.

Khernova, M. G., Bochenkova A. V., & Nemukhin A. V. (2009). Modeling reaction routes from rhodopsin to bathorhodopsin. Proteins, 78, 614-622.

Kleinschmidt, J., & Harosi, F. I. (1992). Anion sensitivity and spectral tuning of cone visual pigments in situ. Proceedings of the National Academy of Sciences of the United States of America, 89, 9181-9185.

Knowles, A., & Dartnall, H. J. A. (1977). The eye: The photobiology of vision. New York: Academic Press.

Kolesnikov, A. V., Tang, P. H., Parker, R. O., 2, C., R.K., & Kefalov, V. J. (2011). The mammalian cone visual cycle promotes rapid M/L-cone pigment regeneration independently of the interphotoreceptor retinoid-binding protein. The Journal of Neuroscience, 31, 7900-7909.

Koskelainen, A., Ala-Laurila, P., Fyhrquist, N., & Donner, K. (2000). Measurement of thermal contribution to photoreceptor sensitivity. Nature, 403, 220-223.

Koskelainen, A., Hemilä, S., & Donner, K. (1994). Spectral sensitivities of short-wavelength and long-wavelength sensitive cone mechanisms in the frog retina. Acta Physiologica Scandinavica, 152, 115-124.

Lamb, T. D., Collin, S. P., & Pugh, E. N. (2007). Evolution of the vertebrate eye: Opsins, photoreceptors, retina and eye cup. Nature Reviews Neuroscience, 8, 960-976.

Lamb, T. D., & Pugh Jr., E. N. (2004). Dark adaptation and the retinoid cycle of vision. Progress in Retinal and Eye Research, 23, 307-380.

Larmuseau, M. H., Vancampenhout, K., Raeymaekers, J. A., Van Houdt, J. K., & Volckaert, F. A. (2010). Differential modes of selection on the rhodopsin gene in coastal Baltic and North Sea populations of the sand goby, *Pomatoschistus minutus*. Molecular Ecology, 19, 2256-2268.

Lewis, P. R. (1955). A theoretical interpretation of spectral sensitivity curves at long wavelengths. The Journal of Physiology, 130, 45-52.

Liebman, P. A., & Entine, G. (1968). Visual pigments of frog and tadpole (*Rana pipiens*). Vision Research, 8, 761-775.

Locket, N. A. (1977) Adaptations to the deep-sea environment. In: Crescitelli, F. (Ed.), The visual system in vertebrates: Handbook of sensory physiology, Berlin-Heidelberg-New York: Springer-Verlag, 67-192.

Loew, E. R., & Lythgoe, J. N. (1978). The ecology of cone pigments in teleost fishes. Vision Research, 18, 715-722.

Lórenz-Fonfría, V. A., Furutani, Y., Ota, T., Ido, K., & Kandori, H. (2010). Protein fluctuations as the possible origin of the thermal activation of rod photoreceptors in the dark. Journal of the American Chemical Society, 132, 5693-5703.

Luo, D. G., Yue, W. W., Ala-Laurila, P., & Yau, K. W. (2011). Activation of visual pigments by light and heat. Science, 332, 1307-1312.

Lythgoe, J. N. (1972). The adaptation of visual pigments to the photic environment. In: Dartnall, H. J. A. (Ed.), Photochemistry of vision: Handbook of sensory physiology. Berlin-Heidelberg-New York: Springer-Verlag, 566-603.

Lythgoe, J. N. (1979). The ecology of vision. Oxford University Press.

Lythgoe, J. N. (1984). Visual pigments and environmental light. Vision Research, 24, 1539-1550.

Lythgoe, J. N. (1988). Light and vision in the aquatic environment. In: Atema, J., Fay, R. R., Popper, A. N., & Tavolga, W. N. (Eds.), Sensory biology of aquatic organisms. New York: Springer, 57-82.

Lythgoe, R. J., & Quilliam, J. P. (1938). The thermal decomposition of visual purple. The Journal of Physiology, 93, 24-38.

MacNichol, E. (1986). A unifying presentation of photopigment spectra. Vision Research, 26, 1543-1556.

Mäkelä, A. V., Heikkilä, O., Kilpeläinen, I., & Heikkinen, S. (2011). ImatraNMR: Novel software for batch integration and analysis of quantitative NMR spectra. Journal of Magnetic Resonance, 211, 186-194.

Mata, N. L., Radu, R. A., Clemmons, R. C., & Travis, G. H. (2002). Isomerization and oxidation of vitamin a in cone-dominant retinas: A novel pathway for visual-pigment regeneration in daylight. Neuron, 36, 69-80.

Mata, N. L., Ruiz, A., Radu, R. A., Bui, T. V., & Travis, G. H. (2005). Chicken retinas contain a retinoid isomerase activity that catalyzes the direct conversion of all-trans-retinol to 11-cis-retinol. Biochemistry, 44, 11715-11721.

Matthews, G. (1984). Dark noise in the outer segment membrane current of green rod photoreceptors from toad retina. The Journal of Physiology, 349, 607-618.

Moon, P. (1961) The scientific bases of illuminating engineering. New York: Dover.

Morel, A. & Smith, R. C. (1974). Relation between total quanta and total energy for aquatic photosynthesis. Limnology and Oceanography, 19, 591-600.

Munz, F. W., & McFarland, W. N. (1973). The significance of spectral position in the rhodopsins of tropical marine fishes. Vision Research, 13, 1829-1874.

Murakami, M. (2008). Crystal structure of squid rhodopsin. Nature, 453, 363-367.

Naka, K. I., & Rushton, W. A. (1966). S-potentials from luminosity units in the retina of fish (Cyprinidae). The Journal of Physiology, 185, 587-599.

Okada, T., Ernst, O. P., Palczewski, K., & Hofmann, K. P. (2001). Activation of rhodopsin: New insights from structural and biochemical studies. Trends in Biochemical Sciences, 26, 318-324.

Okajima, T. I., Wiggert, B., Chader, G. J., & Pepperberg, D. R. (1994). Retinoid processing in retinal pigment epithelium of toad (*Bufo marinus*). The Journal of Biological Chemistry, 269, 21983-21989.

Ovchinnikov, Y. U. A. (1982). Rhodopsin and bacteriorhodopsin: Structure-function relationships. FEBS Letters, 148, 179-191.

Park, J. H., Scheerer, P., Hofmann, K. P., Choe, H., & Ernst, O. P. (2008). Crystal structure of the ligand-free G-protein-coupled receptor opsin. Nature, 454, 183-187.

Parker, R. O., Fan, J., Nickerson, J. M., Liou, G. I., & Crouch, R. K. (2009). Normal cone function requires the interphotoreceptor retinoid binding protein. The Journal of Neuroscience, 29, 4616-4621.

Parry, J. W., Carleton, K. L., Spady, T., Carboo, A., Hunt, D. M., & Bowmaker, J. K. (2005). Mix and match color vision: Tuning spectral sensitivity by differential opsin gene expression in Lake Malawi cichlids. Current Biology, 15, 1734-1739.

Partridge, J. C., Speare, P., Shand, J., Muntz, W. R., & Williams, D. M. (1992). Microspectrophotometric determinations of rod visual pigments in some adult and larval Australian amphibians. Visual Neuroscience, 9, 137-142.

Pegau, W. S., Gray, D., & Zaneveld, R. V. (1997). Absorption and attenuation of visible and near-infrared light in water: Dependence on temperature and salinity. Applied Optics, 36, 6035-6046.

Peskin, J. C. (1957). Concentration of visual purple in a retinal rod of *Rana pipiens*. Science, 125, 68-69.

Provencio, I., & Foster, R. G. (1993). Vitamin A2-based photopigments within the pineal gland of a fully terrestrial vertebrate. Neuroscience Letters, 155, 223-226.

Resek, J. F., Toossi, Z., Wayne, F., Hubbell, L., & Khorana, H. G. (1993). Formation of the meta II photointermediate is accompanied by conformational changes in the cytoplasmic surface of rhodopsin. Biochemistry, 32, 12025-12032.

Reuter, T. (1969). Visual pigments and ganglion cell activity in the retinae of tadpoles and adult frogs (*Rana temporaria* L.). Acta Zoologica Fennica, 122, 1-64.

Reuter, T. E., White, R. H., & Wald, G. (1971). Rhodopsin and porphyropsin fields in the adult bullfrog retina. The Journal of General Physiology, 58, 351-371.

Rieke, F., & Baylor, D. A. (2000). Origin and functional impact of dark noise in retinal cones. Neuron, 26, 181-186.

Sakmar, T. P., Franke, R. R., & Khorana, H. G. (1989). Glutamic acid-113 serves as the retinylidene schiff base counterion in bovine rhodopsin. Proceedings of the National Academy of Sciences of the United States of America, 86, 8309-8313.

Sekharan, S., Altun, A., & Morokuma, K. (2010). Photochemistry of visual pigment in a G_q protein-coupled receptor (GPCR) – insights from structural and spectral tuning studies on squid rhodopsin. Chemistry, 16, 1744-1749.

Shi, Y., Radlwimmer, F. B., & Yokoyama, S. (2001). Molecular genetics and the evolution of ultraviolet vision in vertebrates. Proceedings of the National Academy of Sciences of the United States of America, 98, 11731-11736.

Shichida, Y., Imai, H., Imamoto, Y., Fukada, Y., & Yoshizawa, T. (1994). Is chicken green-sensitive cone visual pigment a rhodopsin-like pigment? A comparative study of the molecular properties between chicken green and rhodopsin. Biochemistry, 33, 9040-9044.

Spady, T. C., Parry, J. W., Robinson, P. R., Hunt, D. M., Bowmaker, J. K., & Carleton, K. L. (2006). Evolution of the cichlid visual palette through ontogenetic subfunctionalization of the opsin gene arrays. Molecular Biology and Evolution, 23, 1538-1547.

Srebro, R. (1966). A thermal component of excitation in the lateral eye of *Limulus*. The Journal of Physiology, 187, 417-425.

St George, R. C. C. (1952). The interplay of light and heat in bleaching rhodopsin. The Journal of General Physiology, 35, 495-517.

Stiles, W. S. (1948). The physical interpretation of spectral sensitivity curve of the eye. Transactions of the Optical Convention of the Worshipful Company of Spectacle Makers. London: Spectacle Makers' Co., 97-107.

Suzuki, T., Makino-Tasaka, M., & Miyata, S. (1985). Competition between retinal and 3-dehydroretinal for opsin in the regeneration of visual pigment. Vision Research, 25, 149-154.

Temple, S. E., Plate, E. M., Ramsden, S., Haimberger, T. J., Roth, W. M., & Hawryshyn, C. W. (2006). Seasonal cycle in vitamin A1/A2-based visual pigment composition during the life history of coho salmon (*Oncorhynchus kisutch*). Journal of Comparative Physiology A, 192, 301-313.

Temple, S. E., Ramsden, S. D., Haimberger, T. J., Veldhoen, K. M., Veldhoen, N. J., Carter, N. L., Roth, W. M., & Hawryshyn, C. W. (2008). Effects of exogenous thyroid hormones on visual pigment composition in coho salmon (*Oncorhynchus kisutch*). The Journal of Experimental Biology, 211, 2134-2143.

Terakita, A. (2005). The opsins. Genome Biology, 6, 213, doi: 10.1186/gb-2005-6-3-213

Tsin, A. T., & Beatty, D. D. (1980). Visual pigments and vitamins A in the adult bullfrog. Experimental Eye Research, 30, 143-153.

Wald, G. (1937). Visual purple system in freshwater fishes. Nature, 139, 1017-1018.

Wald, G. (1939). On the distribution of vitamins A_1 and A_2. The Journal of General Physiology, 22, 391-415.

Wald, G. (1946). The metamorphosis of visual system in amphibia. The Biological Bulletin, 91, 239.

Wald, G. (1968). The molecular basis of visual excitation. Nature, 219, 800-807.

Walls, G. L. (1942). The vertebrate eye and its adaptive radiation. Oxford: Cranbrook Institute of Science, Bulletin No. 19.

Wang, J. S., & Kefalov, V. J. (2011). The cone-specific visual cycle. Progress in Retinal and Eye Research, 30, 115-128.

Ward, J. L., Harris, C., Lewis, J., & Beale, M. H. (2003). Assessment of 1H NMR spectroscopy and multivariate analysis as a technique for metabolite fingerprinting of *Arabidopsis thaliana*. Phytochemistry, 62, 949-957.

Warrant, E. J. (1999). Seeing better at night: Life style, eye design and the optimum strategy of spatial and temporal summation. Vision Research, 39, 1611-1630.

Warrant, E. J. (2004). Vision in the dimmest habitats on earth. Journal of Comparative Physiology A, 190, 765-789.

Whitmore, A. V., & Bowmaker, J. K. (1989). Seasonal variation in cone sensitivity and short-wave absorbing visual pigments in the rudd *Scardinius erythrophthalmus*. Journal of Comparative Physiology A, 166, 103-115.

Wright, A. F., Chakarova, C. F., Abd El-Aziz, M. M., & Bhattacharya, S. S. (2010). Photoreceptor degeneration: Genetic and mechanistic dissection of a complex trait. Nature Reviews Genetics, 11, 273-284.

Wu, Q., Blakeley, L. R., Cornwall, M. C., Crouch, R. K., Wiggert, B. N., & Koutalos, Y. (2007). Interphotoreceptor retinoid-binding protein is the physiologically relevant carrier that removes retinol from rod photoreceptor outer segments. Biochemistry, 46, 8669-8679.

Yeandle, S., (1958). Electrophysiology of the visual system—Discussion. American Journal of Ophthalmology, 46, 82-87.

Yang, K., Farrens, D. L., . Hubbell, W. L., & Khorana, H. G. (1996). Structure and function in rhodopsin. Single cysteine substitution mutants in the cytoplasmic interhelical EF loop region show position-specific effects in transducin activation. Biochemistry, 35, 12464-12469.

Yau, K. W. (1994). Phototransduction mechanism in retinal rods and cones. The Friedenwald Lecture. Investigative Ophthalmology & Visual Science, 35, 9-32.

Yokoyama, S. (2002). Molecular evolution of color vision in vertebrates. Gene, 300, 69-78.

Yokoyama, S., & Yokoyama, R. (1996). Adaptive evolution of photoreceptors and visual pigments in vertebrates. 27, 543-567.

Yoshizawa, T. (1972). The behaviour of visual pigments at low temperatures. In: Dartnall, H. J. A. (Ed.), Photochemistry of vision: Handbook of sensory physiology. Berlin-Heidelberg-New York: Springer-Verlag, 147-179.

Zhukovsky, E. A., & Oprian, D. D. (1989). Effect of carboxylic acid side chains on the absorption maximum of visual pigments. Science, 246, 928-930.