Molecular Tuning of Rhodopsins for High Visual Sensitivity in Different Light Environments: Variation in Absorbance Spectrum and Opsin Sequence within and between Species

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Academic Dissertation

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Helsinki 2009
To my family,
List of publications:

This thesis is based on the following four publications, which will be referred to in the text by the Roman numerals I-IV:


* These authors contributed equally to the work
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List of abbreviations

ATP  adenosine triphosphate
A1  visual pigments with retinal as chromophore
A2  visual pigments with 3, 4-dehydroretinal as chromophore
A3  visual pigments with 3-hydroxyretinal as chromophore
A4  visual pigments with 3, 4-dehydroxyretinal as chromophore
cDNA  complementary deoxyribonucleic acid
cGMP  cyclic guanosine monophosphate
CNG-channel  cyclic nucleotide-gated channel
DNA  deoxyribonucleic acid
DOM  dissolved organic matter
EMBL  European Molecular Biology Laboratory
ERG  electroretinogram
GDP  guanosine diphosphate
GMP  guanosine monophosphate
G-protein  guanylate nucleotide-binding protein
GPCR  G-protein coupled receptors
GTP  guanosine triphosphate
IP3  inositol triphosphate
kDa  kiloDalton
LWS  long-wavelength sensitive
λmax  wavelength of maximum absorbance
M-cone  middle-wavelength sensitive cone
MSP  microspectrophotometry
MWS  middle-wavelength sensitive
N  noise
OD  optical density
OS  photoreceptor outer segment
PCR  polymerase chain reaction
PDE  phosphodiesterase
PIP2  phosphatidyl inositol diphosphate
PLC  phospholipase C
QC  quantum catch
Rh  rhodopsin
RH1  rhodopsin
RH2  RH1 like
rk 1, 2, 3  rhodopsin kinases
S-cone  short-wavelength sensitive cone
SD  standard deviation
SEM  standard error of means
SNR  signal-to-noise ratio
SWS1  short-wavelength sensitive type1
SWS2  short-wavelength sensitive type
Amino acids

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Abstract

Visual pigments of different animal species must have evolved at some stage to “match” the prevailing light environments, since all visual functions depend on their ability to absorb available photons and transduce the event into a reliable neural signal. There is a large literature on correlation between the light environment and spectral sensitivity between different fish species. However, little work has been done on evolutionary adaptation between separated populations within species. More generally, little is known about the rate of evolutionary adaptation to changing spectral environments.

The objective of this thesis is to illuminate the constraints under which the evolutionary tuning of visual pigments works as evident in: scope, tempo, available molecular routes, and signal/noise trade-offs. Aquatic environments offer “Nature’s own laboratories” for research on visual pigment properties, as naturally occurring light environments offer an enormous range of variation in both spectral composition and intensity. The present thesis focuses on the visual pigments that serve dim-light vision in two groups of model species, teleost fishes and mysid crustaceans. The geographical emphasis is in the brackish Baltic Sea area with its well-known postglacial isolation history and its aquatic fauna of both marine and fresh-water origin.

The absorbance spectrum of the (single) dim-light visual pigment were recorded by microspectrophotometry (MSP) in single rods of 26 fish species and single rhabdoms of 8 opossum shrimp populations of the genus *Mysis* inhabiting marine, brackish or freshwater environments. Additionally, spectral sensitivity was determined from six *Mysis* populations by electroretinogram (ERG) recording. The rod ops in gene was sequenced in individuals of four allopatric populations of the sand goby (*Pomatoschistus minutus*). Rod opsins of two other goby species were investigated as outgroups for comparison.

Rod absorbance spectra of the Baltic subspecies or populations of the primarily marine species herring (*Clupea harengus membras*), sand goby (*P. minutus*), and flounder (*Platichthys flesus*) were long-wavelength-shifted compared to their marine populations. The spectral shifts are consistent with adaptation for improved quantum catch (QC) as well as improved signal-to-noise ratio (SNR) of vision in the Baltic light environment. Since the chromophore of the pigment was pure A1 in all cases, this has apparently been achieved by evolutionary tuning of the opsin visual pigment. By contrast, no opsin-based differences were evident between lake and sea populations of species of fresh-water origin, which can tune their pigment by varying chromophore ratios.

A more detailed analysis of differences in absorbance spectra and opsin sequence between and within populations was conducted using the sand goby as model species. Four allopatric populations from the Baltic Sea (B), Swedish west coast (S), English Channel (E), and Adriatic Sea (A) were examined. Rod absorbance spectra, characterized by the wavelength of maximum absorbance (λ_max), differed between populations and correlated with differences in the spectral light transmission of the respective water bodies. The greatest λ_max shift as well as the greatest opsin sequence difference was between the Baltic and the Adriatic populations. The significant within-population variation of the Baltic λ_max values (506-511 nm) was analyzed on the level of individuals and was shown to correlate well with opsin sequence substitutions. The sequences of individuals with λ_max at shorter
wavelengths were identical to that of the Swedish population, whereas those with $\lambda_{\text{max}}$ at longer wavelengths additionally had substitution F261F/Y in the sixth transmembrane helix of the protein. This substitution (Y261) was also present in the Baltic common gobies and is known to redshift spectra. The tuning mechanism of the “long-wavelength type” Baltic sand gobies is assumed to be the co-expression of F261 and Y261 in all rods to produce $\approx 5$ nm redshift. The polymorphism of the Baltic sand goby population possibly indicates ambiguous selection pressures in the Baltic Sea.

The visual pigments of all “lake” populations of the opossum shrimp (Mysis relicta) were red-shifted by 25 nm compared with all Baltic Sea populations. This is calculated to confer a significant advantage in both QC and SNR in many humus-rich lakes with reddish water. Since only A2 chromophore was present, the differences obviously reflect evolutionary tuning of the visual protein, the opsin. The changes have occurred within the ca. 9000 years that the lakes have been isolated from the Sea after the most recent glaciation. At present, it seems that the mechanism explaining the spectral differences between lake and sea populations is not an amino acid substitution at any other conventional tuning site, but the mechanism is yet to be found.

**Keywords:** visual pigment, visual ecology, rod opsin sequence, spectral sensitivity, light adaptation, signal-to-noise ratio, quantum catch
1 Introduction

Vision is indisputably the sense that provides most information about the environment for most vertebrates as well as many groups of arthropods and molluscs. These animals have visual cells and eyes that allow high spatio-temporal resolution, often wavelength discrimination, and in some cases polarization discrimination. The solutions within these animal groups show enormous diversity, especially with respect to eye design. Moreover, in highly visual animals large parts of the brain are involved in processing and utilizing the visual information. Advanced vision has evolved to extract biologically relevant information from light, mainly that reflected from objects in the environment. As vision is then the main provider of information about threats and opportunities in the habitat, it largely drives behaviour in these species.

At the most basic level, vision requires that light acts on receptor molecules to produce a biochemical and finally electrical signal in a photoreceptor cell. Good photon catch is a primary prerequisite for efficient vision. The photon-catching molecules are membrane receptor proteins called visual pigments or rhodopsins, and these are basically of the same type across the animal kingdom. They determine the wavelength domain of electromagnetic radiation that is accessible for seeing. Light-sensitivity is conferred by a vitamin A-derived cofactor (“chromophore”) that can be isomerized by light and is covalently bound to the receptor protein, the “opsin”, via a Schiff base linkage. Isomerization of the chromophore results in a conformational change in the opsin, which initiates a transduction cascade ending in a change of the membrane potential of the photoreceptor cell. Interactions between the dipolar and electrostatic environment of the chromophore and the amino acid residues of the opsin protein determine the wavelength band of light that can activate a visual pigment (Nathans et al 1989, Sakmar et al 1989, Yokoyama 2002).

The present thesis is concerned with the performance of visual pigments that serve vision in very dim light. It is a trivial truth that dim light visual pigments must in some sense spectrally “match” the prevailing light in the respective habitats. The study of correlations between the light environment and spectral sensitivity has been a mainstay in vision research (Lythgoe 1972, Bowmaker et al 1994, Yokoyama 2000, Pointer et al 2007). The primary selective pressure on visual pigments seems to be the spectral range and intensity of the prevailing light (Bowmaker and Hunt 2006, Bowmaker 2008) especially in underwater environments. The majority of the earth (70 %) is covered by oceans and lakes. The optical properties of the water vary widely depending on the amount of organic and inorganic matter, and the intensity and the spectral range of the light at different depths will differ correspondingly between diverse aquatic light environments. This challenges the visual capabilities of aquatic animals. Most species need good dim light vision, being active in photon scarce conditions at least part of their lives. Deep in seas or lakes, the surrounding light is almost
monochromatic and it is generally useless to have more than one visual pigment, residing in a single class of photoreceptor cells. Such dim light receptors are represented by the rods of vertebrates and the rhabdoms of many invertebrates. When analyzing their performance, it is necessary to take into account also the thermal noise that is inevitably connected with each step of the visual process. Only those signals that differ statistically significantly from the noise can be detected. The signal-to-noise ratio (SNR) is a measure of the detectability of the signal. Thus dim-light visual pigments should not only catch as many as possible of the scarce photons (which requires accurate spectral tuning), but they should also be as resilient as possible against “spontaneous” activation by thermal energy alone, which will trigger noise events that are identical to real photon signals. The greater the number of photon absorptions and the lower the rate of thermal activations, the greater will be the signal relative to the noise and the more reliable is the visual discrimination.

Altogether, an enormous diversity of visual systems has evolved in different animals by variations in the design of eyes, neural processing networks, photoreceptor cells, and visual pigments. Yet the operation of all can be analyzed based on a few fundamental principles. The possibility to integrate the extensive data obtained from ecology, physiology, behavior, molecular biology and biophysics is a true benefit of vision science. The puzzle to resolve the basic elements related to the vision of the vertebrates and invertebrates is still unfinished. The objective of this thesis is to illuminate the constraints under which the evolutionary tuning of dim light visual pigments works (tempo, scope, available molecular routes, signal/noise trade-offs) by using aquatic animal models: fishes and shrimps. The concept of optimality of such pigments is also assessed.
2 Background and review of the literature

2.1 Animal eyes: great diversity

During the history of the earth, sunlight has been the major force driving the evolution of the living organisms. It is of course the ultimate source of almost all energy available for life, but in addition, the information carried by light has been utilized from a very early stage in evolution, starting with a class of sensory receptor proteins, “type 1 opsins”, in protobacteria some 3 billion years ago. In higher animals, the sensory structures that serve the use of light information comprise three main levels: (1) receptor molecules (rhodopsins) that undergo a conformational change upon absorbing photons; (2) photoreceptor cells where this event is transduced into an electrical nerve signal; (3) organs with different types of apertures or optics guiding light to the underlying matrix of photoreceptor cells (Figure 1). Such an organ is an eye. Eyes have evolved in many shapes, sizes and designs numerous times independently, although subject to a few common underlying principles.

Due to the physics of light, there are relatively few ways to construct an eye that gives a functional image. Most of these structures have evolved more than once independently (Land and Fernald 1992). More fundamentally, ancient biochemical solutions are retained at the cellular level - the evolution of widely different eyes encompasses considerable homology among structural and developmental molecules, while, on the other hand, vertebrates and invertebrates may possess similar eye designs although they are not originated from a common ancestor (convergent evolution). The use of homologous genes to build nonhomologous structures is essential in understanding the evolution of the eye (Fernald 2006).

The main tasks of eyes are to collect light efficiently and ideally, ensure that the spatial distribution of light on the underlying photoreceptor matrix to some extent reproduces the spatial distribution in the environment. The variation of the optical design of the eye generally expresses a trade-off between light sensitivity and spatial resolution and is strongly related to the environment and mode of life of the specific animal. The conventional division of eyes is into “simple” i.e. single-chambered or camera-like eyes, and “compound” eyes (Figure 1). The topological solutions related to the image forming are different in these groups, “concave” and “convex”, respectively (Land and Fernald 1992). Of the animal groups studied in the present thesis, fishes have “concave”, single-chambered eyes, and mysids have “convex”, compound eyes.

Almost all vertebrates have camera-like eyes with similar optical and anatomical construction (Land and Fernald 1992). The ray of light passes through the cornea, the anterior and the posterior chamber, then through the lens, the vitreous body and finally it reaches the retina and its specialized cell layers (ganglion cells, amacrine cells, bipolar cells, horizontal cells, and photoreceptor
Fig. 1 All animal eyes have a homologous photoreceptor molecule, opsin. The diverse speciation for housing large amounts of visual pigment and for transducing the information into electrical signals appear to be nonhomologous (although many transcription factors and other developmental molecules are shared). Instead they are parallel solutions to common problems of sensitivity and speed of visual receptor cells. The different types of photoreceptor cells have subsequently been recruited many times independently to produce imaging eyes. The original belief that vertebrates and invertebrates each had their own type of photoreceptor cell is abandoned since both receptor types are present in both vertebrates and invertebrates. The figure is modified from Land and Nilsson (2002).

cells with light detecting molecules). As the photoreceptor cells are situated in the most distal layer, the light has to pass through all retinal cell layers before being detected. In terrestrial animals most of the light refraction occurs at the cornea, at the interface of air and water. Conversely, in aquatic animals the water/cornea interface has little refractive power and the refraction occurs in the lens. Therefore, fishes and amphibians adjust the distance of the lens and the retina to achieve a focused image; they do not possess the ability to adjust the thickness of the lens as most of the terrestrial vertebrates do.

The compound eyes can be basically divided into two main types: (1) apposition eyes and (2) superposition eyes. (1) Diurnal insects and crustaceans possess the classical apposition eye, in which the erect image is built up from the elementary contributions of all the separate ommatidia and single photoreceptors (Land and Fernald 1992). The apposition eye is the best-known and most common of all compound eyes,
consisting of ommatidia with lens, light guiding structures and rhabdom (for more details, see Land and Fernald 1992). (2) In superposition eyes of many nocturnal insects and crustaceans, the rhabdoms do not lie immediately behind the facet lenses, but forms a layer of cells much deeper with a zone of clear material that separates them from the optics (Land and Fernald 1992). The retina forms a single sheet, which is not broken up into discrete ommatidial units as in apposition eyes. The parallel light that enters a group of corneal facet lenses is focused onto a single rhabdom (Lythgoe 1972). A superposition eye provides two-three orders of magnitude more light to the photoreceptors than an apposition eye of the same size. Therefore in dim light conditions superposition eyes predominate (Land 1999) although apposition eyes are also found in several nocturnal insects (Warrant et al 2004, Warrant 2008). Apposition and superposition eyes are further divided into different groups according to their special structures (Land and Nilsson 2002, Fernald 2006).

### 2.2 Photoreceptor cells are basically of two kinds

Photoreceptor cells, located in the retina or other structure serving vision, are key components of the visual systems of the animals. They are highly specialized cells which capture photons and transduce the information that a photon has been received into a neural signal. Photoreceptors carry the receptor molecules, rhodopsins or visual pigments, in their membranes. To accommodate a great amount of visual pigment and thus ensure efficient absorption of photons, the total surface of photoreceptor membranes is generally enormously increased by invaginations or microvilli. By photon catch, by the amplification and the noisiness of the transduction machinery, and by the temporal properties of their electric response, the photoreceptor cells set strong constraints on visual sensitivity and the temporal resolution of vision.

Two fundamentally different constructions have emerged during evolution: the rhabdomeric and the ciliary photoreceptors (Figure 1 and Figure 2). Of the animal groups used in the present thesis, the mysid shrimps have the former type and the fishes the latter type of photoreceptor cells.

To make a simplifying generalization, ciliary photoreceptors are typical of the vertebrates whereas rhabdomic photoreceptors are present in invertebrates. These photoreceptor types have two distinctive features: (1) the topology of the membrane for photopigment storage: rhabdometric photoreceptors have apical microvilli whereas the invaginated membrane of ciliary photoreceptors is derived from a modified cilium, and (2) the steps in the signal transduction cascade after the G-protein is bound (Osorio and Nilsson 2004). Ciliary photoreceptors use a signalling pathway where a phosphodiesterase (PDE) is activated and the concentration of cyclic guanosine monophosphate (cGMP) is reduced in the outer segment of the cell (Figure 2). In rhabdomeric photoreceptors signal
transduction involves activation of phospholipase C (PLC) and the inositol phosphate (IP3) pathway (Arendt 2003, Arendt et al 2004). Both of these pathways (IP3 and PDE) exist in cell signalling in all animals; the dissimilarity is that they are used differently in the different photoreceptors. Recently, Arendt and his associates (2004) showed that ciliary and rhabdomeric photoreceptors use opsins of different subfamilies opsin: c-opsins are present exclusively in ciliary photoreceptors and r-opsins in rhabdomeric photoreceptors. The G-proteins also represent different subfamilies (Gi and Gq, respectively).

Until recently, it was widely thought that ciliary photoreceptors are present exclusively in the superphylum deuterostomes (including vertebrates) whereas rhabdomeric photoreceptors are present solely in the superphylum protostomes (comprising most invertebrates). However, it is now clear that both types coexist in a variety of organisms; in the scallop (Miller 1958), in the polychaete worm (Arendt et al...
2.2.1 Ciliary photoreceptors: vertebrate rods and cones

Most vertebrates have a duplex retina with two types of ciliary photoreceptors: rods and cones. As usual, exceptions to this rule are also found: for example, the nocturnal Tokay gecko (Gekko gecko) and the diurnal American chameleon (Anolis carolinensis) have pure-rod retinas and pure-cone retinas, respectively (Yokoyama 2000). More than 140 years ago Schultze (1866, 1867 reviewed in Crescitelli 1972) suggested that photoreceptors are designed for different light intensities; rods are made for dim-light conditions (scotopic vision) and cones are for bright light conditions (photopic vision). This is generally called the Duplicity Theory of Vision (Crescitelli 1972).

Rod and cone photoreceptors are specialized for different aspects of vision. Rods are focused on high sensitivity at the expense of spatial and temporal resolution and the cones are focused on resolution at the expense of sensitivity (Rodieck 1998). Cones are typically 100 times less photosensitive than rods and their response kinetics are several times faster (Baylor 1987). In addition to handling high light intensities the cone system enables colour vision. Mechanisms for light detection in animals. Although the structure and the transduction cascades are different, ciliary and rhabdomeric photoreceptors both have G-protein-coupled rhodopsins for catching photons and initiating transduction (Arendt 2003). Since the visual pigment in a single cone type does not discriminate the wavelength of light (principle of univariance: the wavelength information is lost as soon as the photon is absorbed; Naka and Rushton 1966) at least two different cones with different spectral sensitivities are needed for proper colour vision. In essence, colour vision is based on the comparison of the quantum catch of one photoreceptor class versus the quantum catch of another class. In the actual colour discrimination the signal processing of downstream neurons (bipolar cells, ganglion cells) is needed. The most efficient number of cone types for colour vision seems to be three. Adding a fourth spectral class probably provides little or no advantage in hue discrimination (Barlow 1982, Osorio and Vorobyev 2008) but may improve colour constancy.

Vertebrate photoreceptor types (rods and cones) can often be distinguished by their size and shape (from which they derive their names), and the arrangement of the membranous disks in the outer segments. The most basic difference, however, concerns the type of visual pigment and the enzymes and other proteins involved in phototransduction. Rods and cones also differ with respect to the distribution across the retina, and the patterns of their synaptic connections (Loew and Lythgoe 1978, Rodieck 1998).
The photoreceptors consist of three major parts: outer segment (OS) with membrane disks for the visual pigment storage, inner segment and soma for the energy supply and protein synthesis, and the synaptic body for signal transmission. In the retina rod photoreceptors are generally more abundant than cones (in the human retina 120 million vs. 6 million). However, the information from many rods is pooled to retinal ganglion cells in order to obtain higher sensitivity of rod vision. In the human eye, thousands of rods may contribute to one pool. The cost of investing in a duplex system is decreased by the fact that rods are energetically cheaper to maintain than the cones as measured by adenosine triphosphate (ATP) consumption in light (Okawa et al 2008). This also explains why rods are allowed to outnumber cones even in diurnal animals (Okawa et al 2008). In the cylindrical rod OSs, the membrane disks are completely separated from the plasma membrane and are “floating” within the cell. The cone OSs are smaller, with a generally shorter and more tapering outer segment, where the disks are invaginations of the plasma membrane and in direct connection with the extracellular space (Kusmic and Gualtier 2000).

The retinal distribution of photoreceptors is generally uneven. Normally the peak density of cones is located in the retinal centre and the cell density is lower in the peripheral retina. In many species cones are concentrated an area of acute vision (fovea centralis, a spot of maximal acuity and colour sensitivity) or in a visual streak (band-shape increase in retinal thickness or increase in photoreceptor/ganglion cell density) whereas the peripheral retina is rod-dominated (Collin 1999). The interspecies variation in retinal cell types and distribution is extensive (Britt et al 2001, Ebrey and Koutalos 2001, Reckel et al 2002). The distribution of cells in vertebrates varies with developmental stage, especially in fish and amphibians during maturation from larval to adult forms (Partridge and Cummings 1999, Ebrey and Koutalos 2001, Miyazaki et al 2008).

It is often assumed that one single visual pigment is associated with a single type of photoreceptor and generally this seems to be true. However, there are reports of photoreceptors that contain traces of a second pigment (Röhlich et al 1994, Ahnelt and Kolb 2000, Applebury et al 2000). In small rodents, cones coexpress both short wavelength and longer wavelength sensitive opsin genes (S- and M-cones) throughout the retina (Röhlich et al 1994, Lukats et al 2002). One advantage may be that coexpression broadens the spectral range of a cone especially towards the short-wavelength domain.

The phototransduction cascade initiated by the absorption of a photon has been studied in great detail in several vertebrate species (Cooper 1979, Shichida and Imai 1998, Pugh and Lamb 2000). The initial event is activation of a visual-pigment molecule by isomerisation of the chromophore. The end result is a change of the membrane potential (hyperpolarization) of the photoreceptor cell leading to a decrease in the release of neurotransmitter, glutamate, in the first synapse onto bipolar and horizontal cells. In between
is a biochemical amplification cascade where activation of the G-protein transducin activates a phosphodiesterase (PDE), which then hydrolyzes molecules of the internal transmitter cGMP into GMP. The drop in the cytoplasmic cGMP concentration leads to the closure of cGMP-gated cation channels in the plasma membrane of the OS. As the inward current (carried by sodium and to a lesser extent by calcium) is decreased, the cell is hyperpolarized. In a dark-adapted rod, activation of a single molecule of visual pigment may lead to hydrolysis of 10000 molecules of cGMP and an electrical response amounting to 3-6 % of the maximal response. Thus a rod can signal the absorption of single photons reliably.

2.2.2 Rhabdomeric photoreceptors: invertebrate rhabdoms

Rhabdomeric photoreceptors have been described in arthropods (Chelicerata, Crustacea, Myriapoda, Hexapoda), molluscs, annelids and flatworms. They are found in simple and compound eyes as well as isolated eye spots having no optical resolving power (Land and Nilsson 2002). Most insects and crustaceans have compound eyes composed of a few thousand separate light-receptive units or ommatidia, each of which consists of a corneal facet/lens and a central rhabdom (from Greek *rhabdos*, rod). The rhabdoms are made up of the microvillar arrays, rhabdomeres of several photoreceptor cells, which are juxtaposed in adjacent cells (Miller 2005). The rhabdom is the region with high visual pigment concentration and is responsible for the light sensitivity. In the rhabdoms, microvilli are positioned to maximize light absorption. The membrane surface area is further increased by throwing up their apical surfaces into numerous folds. There is a similar principle of construction as in vertebrate rods and cones, although the photoreceptive membraneous compartments are less clearly separated from the cell body of the photoreceptor. The photoreceptor cells are placed in a bundle and the microvilli arranged towards the center of this cylindrical structure.

The membrane potential of these microvillar photoreceptors depolarizes in response to light, the opposite response to that of vertebrates rods and cones, which hyperpolarize upon illumination. As in rods and cones, transduction is initiated by the interaction between rhodopsin and a G-protein. In microvillar receptors, this triggers the PLC pathway with several reaction products that are candidates for roles as internal transmitters of varying importance between groups. The final outcome is opening of cation channels (transient receptor potential-channels, TRP) in the plasma membrane and/or liberation of calcium from internal stores. Both lead to a massive increase in internal calcium on somewhat different time scales (liberation from internal stores being slower). The dominant transduction pathway varies between groups and is functionally related to the temporal resolution required by the animal’s lifestyle. Especially notable are the extremely fast responses achieved in fly photoreceptors thanks to TRP channel
properties, the small volume of the microvillar compartments and physical coupling of the main phototransduction molecules into functional assemblies (Hardie and Postma 2008).

2.2.3 Photoreceptor cells in fishes and aquatic crustaceans

The species studied in this thesis represent, on one hand, teleost fishes, on the other hand amphipod crustaceans. The common feature is that both groups occasionally need to see in very dim light spectrally limited by the transmittance of different water bodies, and both possess a highly sensitive single-pigment photoreceptor system for this task.

Most teleost fishes have many types of cone cells in addition to the rods. Principally, the number of photoreceptor types, their sensitivity and their arrangement within the retina correlate with the spectral characteristics of the habitat: the width of the light spectrum and the amount of the light available (Bowmaker 1995). Many diurnal fishes, especially those living near the surface, show a well-developed retina with many different cone types (Engström 1963), suggesting trichromatic or tetrachromatic color vision (Douglas and Partridge 1997). Nevertheless, fishes living in deeper regions or more restricted spectral environments are often limited to one or two spectral classes of cone (Lythgoe 1972, Bowmaker 1990, Bowmaker et al 1994) or only one photoreceptor cell type like in many deep-sea fishes (Denton and Warren 1957, Wald et al 1957, Munz 1958, Beatty 1969, Crescitelli et al 1985, Partridge et al 1988, 1989, 1992, Crescitelli 1991, Douglas et al 1995, Douglas and Partridge 1997). Like most vertebrates, the fish species studied here possess only one type of rods and colour vision is therefore not possible in very dim light. The task of their rod system is to provide high absolute light sensitivity. However, many amphibians and some deep-sea fishes (Douglas et al 1998) have two or even three types of cells that morphologically viewpoint are rods, theoretically enabling scotopic colour vision.

The Mysis relicta group of species has only one spectral class of photoreceptor cells with a single visual pigment and their vision seems to have evolved to ensure high light sensitivity in rather simple tasks. The simplicity is not due any limitation inherent in crustaceans, but correlated with the behaviour and the photic environment of the particular species. Like vertebrates, many crustaceans have several spectrally different photoreceptors and excellent colour vision. The extreme example are the stomatopods (mantis shrimps; Marshall 1988, Cronin and Marshall 1989, Marshall et al 2007), which have the highest number of different spectral classes of photoreceptors in any known animal group.
2.3 All visual pigments are G-protein-coupled receptors

2.3.1 Opsin plus chromophore: a photosensitive molecule

The light sensitivity of the photoreceptors is mediated by the visual pigment molecules lying within the disk membranes of the photoreceptor outer segments in vertebrates or in the rhabdoms of invertebrates. The main part of the visual pigments of all animals is a 7-transmembrane (7-TM) protein belonging to the opsin superfamily of G-protein coupled receptors (GPCRs) to which a light-sensitive “chromophore” (retinal) is covalently bound. The visual pigment is the photosensitive molecule that initiates the process which finally leads to an electric response of the cell. The visual pigments have bell-shaped absorbance spectra of basically constant shape and can therefore be easily characterised by their wavelength of maximum absorption ($\lambda_{\text{max}}$) describing the position of the absorbance spectrum on the wavelength axis. The constancy of shape implies that it is possible to construct nomograms allowing the entire absorption curve of any visual pigment to be calculated from the $\lambda_{\text{max}}$ of the pigment (Dartnall 1953, Partridge and DeGrip 1991, Hárosi 1994, Govardovskii et al 2000). The $\lambda_{\text{max}}$ values of known visual pigments vary from around 350 nm in the UV to 635 nm in the far red (Bowmaker 1990, 1995, Bowmaker et al 1991).

2.3.2 General structure of opsin and chromophore

The visual pigments consist of two main components: 1) an apoprotein part, opsin, and 2) prosthetic group, called chromophore. Opsins are member of an extremely large superfamily of integral membrane protein, the G-protein coupled receptors (GPCRs). This group comprises approximately 6% of the humane genome and takes part in a diverse array of physiological processes in vertebrates, including neurotransmission, memory, learning, and various endocrine and hormonal pathways. They all share the same tertiary structure, mechanisms of activation, and activation of G-protein although the effectors in the cascade may differ (Filipek et al 2003). The opsin family consists of seven functionally different subfamilies according to molecular phylogeny (Figure 3; Yokoyama and Yokoyama 2000, Terakita 2005, Fernald 2006). The invertebrate opsins are further classified into four major groups (Yokoyama and Yokoyama 2000). The vertebrate visual and non-visual opsin subfamily includes five visual and one non-visual opsin protein. RH1 pigments are usually expressed in rod photoreceptor cells and the other four classes of visual opsins in cone photoreceptor cells. The origin of these five groups of pigments seems to be very old. They were present already prior to the divergence of the major vertebrate groups (Yokoyama and Yokoyama 1996).

Opsins are integral transmembrane proteins composed of a single,
Fig. 3 Opsi phylogeny. (A) A simplified schematic molecular phylogenetic tree inferred by the neighbour-joining method showing the seven known opsin subfamilies according to the functional classification. The tree and the names of the opsin subfamilies are modified from Terakita (2005) and Fernald (2006). Three opsin subfamilies transduce light using G protein-coupled mechanisms (G_q, G_o, G_o); the best known are invertebrate G_q – coupled opsins (r-opsins) and vertebrate G_o – coupled opsins (c-opsins). Encephalopsin and its teleost homolog tmt are found in multiple tissues with unknown function. Neuropsins are found in eye, brain, testes, and spinal cord in mouse and human, but little is known about them. Peropsins and the photoisomerase family of opsins bind to all-trans-retinal, and light isomerizes it to the 11-cis form, which suggests a role in photopigment renewal. These are expressed in tissues adjacent to photoreceptors, consistent with this role. (B) Invertebrate opsins can be classified into four major groups: Rh1/2/6 group, Crab opsins (including Mysis relicta), Rh3/4/5 group and mollusc group (Yokoyama and Yokoyama 2000). (C) Vertebrate opsins are divided into six groups: RH1 (rhodopsins, \( \lambda_{\text{max}} \approx 480-510 \) nm), RH2 (\( \lambda_{\text{max}} \approx 450-530 \) nm), SWS1 (\( \lambda_{\text{max}} \approx 360-440 \) nm), SWS2 (\( \lambda_{\text{max}} \approx 400-450 \) nm), LWS/MWS group (\( \lambda_{\text{max}} \approx 510-560 \) nm), and P/non visual (\( \lambda_{\text{max}} \approx 470-480 \) nm) (Yokoyama and Yokoyama 2000, Terakita 2005). The primordial retinal opsin of vertebrates diverged into long-wave-sensitive and short-wave sensitive branches and then later split into several subgroups. The RH1 pigment seems to represent the most recent development among these classes and is expressed in vertebrate rod photoreceptors.

A polypeptide chain, consisting in most animal species of about 340-390 amino acids (Shichida and Imai 1998, Gärtner 2000, Palczewski et al 2000) although the squid Todarodes has 448 amino acids (Murakami and Kouyama 2008,
Shimamura et al 2008; Figure 4) The calculated molecular mass is approximately 30-50 kDa (Terakita 2005, Murakami and Kouyama 2008, Shimamura et al 2008). Structural studies of rhodopsin and its biochemical and biophysical properties have been extensively reviewed (Sakmar et al 2002, Palczewski 2006, Lodowski et al 2009). The opsin polypeptide folds into seven highly hydrophobic membrane-spanning α-helices connected by hydrophilic loops. The interior of the helix bundle forms a binding cavity for the chromophore which is covalently linked to the protein via a protonated Schiff base to a lysine residue from the seventh helix (bovine numbering system: site 296; Hargrave et al 1984, Hargrave and McDowell 1992). In vertebrate rods, the extracellular parts of the opsin – the N-terminal domain and the extracellular loops – are in the enclosed intradiscal space, while the C-terminal region and the intracellular loops face the cytoplasm. Although the opsincs vary in the number and sequence of amino acids, the structure with transmembrane helices and the cytoplasmic loops are highly conserved. For the interspecies comparisons the numbering system of each amino acid is adopted from the mammalian, bovine, rod opsin first modelled by Hargrave et al (1983, 1984).

The first crystal structure of an invertebrate rhodopsin, that of the squid Todarodes pacificus, has recently been determined (Murakami and Kouyama 2008; Shimamura et al 2008). The invertebrate opsins belong to a Gα subfamily of GPCR. The structure and presumably the function are divergent in certain parts of the molecule compared to the vertebrate (bovine) opsin. The arrangement of helices I to VIII is similar to the vertebrate opsins, but the transmembrane helices V and VI are longer and protrude farther into the cytoplasm. The additional helix IX locates at the cytoplasmic part of molecule. The squid opsin is larger than vertebrate rhodopsins due to the extension in the carboxyl terminal part of the protein. The amino-acid residues in contact with retinal and the orientation of retinal within the protein are different than previously reported in bovine rhodopsin; the Tyr111, Asn87, and Asn185 residues are located within hydrogen-bonding distances from the nitrogen atom of the Schiff base and Lys305 is bound to retinal (Shimamura et al 2008).
The chromophore part of the pigment molecule consists of a long chain of carbons with alternating single and double bonds and a β-ionone ring. The chromophore in vertebrates is derived from vitamin A1 (retinal) or vitamin A2 (3, 4-dehydroretinal) as in some fish (especially fresh water species), amphibians, aquatic reptiles, but also in some invertebrates including the Mysis species studied here (Knowles and Dartnall 1977; IV, Figure 4). The difference between A1 and A2 vitamins is the additional carbon-carbon double bond in the β-ionone ring of the A2 chromophore, inducing the shift of the absorbance spectra towards longer wavelengths. The shape of the spectra also becomes wider and the peak becomes lower (Bridges 1967). Retinal and 3, 4-dehydroretinal can exist in several isomeric forms, of which only two, 11-cis and all-trans are important in the natural visual process. Absorption of a photon isomerizes the molecule: the bond between the 11th and 12th carbon atoms straightens and the configuration of the molecule alters from cis to trans geometry. Additionally, hydroxylated forms of retinal have been found in insects (A3, 3-hydroxyretinal, Vogt and Kirschfelt 1984, Gärtner 2000) and in some cephalopods (A4, 4-hydroxyretinal, Matsui et al 1988, Seidou et al 1990, Gärtner 2000). All visual pigments with retinal as their chromophore are known as rhodopsins, while those using vitamin 3, 4-dehydroretinal are often referred to as porphyropsins and hydroxylated retinal-based chromophores as xanthopsins, which are far more polar than rhodopsin and porphyropsins.

2.3.3 Functional variables of visual pigment activation: spectral sensitivity and thermal stability

All information available for vision is based on photons absorbed by visual pigment and transduced into an electrical signal in the photoreceptor cell. Once a photon has activated the pigment molecule, the information about its energy (the wavelength of the light) is lost – the quantal response of the cell is standardized (“the principle of univariance”), although subject to some random variation in shape and size. The signals may subsequently be processed in many ways, e.g., pooled and filtered in space and time, thresholded, or coupled antagonistically, but no information about the visual environment is added beyond the primary pattern of visual-pigment activation in the retina. Therefore, the functional properties of the visual pigment are crucially important for all vision.

Some basic properties of pigments are remarkably constant, suggesting that they have effectively been optimized by evolution. This is true of peak absorbance as well as the so-called quantum efficiency of isomerisation, i.e., the proportion of absorbed photons that trigger a visual signal (ca. 2/3; see Dartnall 1968). Peak sensitivity varies only between pigments using different chromophores: the photosensitivity of A2 pigments is only about 70% that of A1 pigments (Dartnall 1972).

There are two main variables associated with the activation of visual pigments. Other variables have to do
with deactivation and the subsequent restoration of pigment responsivity. The most important activation variable is spectral sensitivity, i.e., the position of the absorbance spectrum on the wavelength scale. Since the primary requirement for vision is good photon catch, it is natural that the absorbance spectrum should be “aligned” with the spectral environment where the animal lives. Spectral tuning generally means adjusting the energy barrier for isomerisation (Barlow 1957, Ala-Laurila et al 2004b). For example, red-shifting a pigment means lowering the barrier, thus increasing the probability that the energy carried by “red”, low-energy photons should suffice to isomerise the chromophore. Hence, within a certain class of pigments, there will be an inverse correlation between the wavelength of maximum absorbance and the activation energy.

The second main variable is the pigment’s tendency to be activated “spontaneously” in the absence of any light, by thermal energy alone (Autrum 1943, Barlow 1956). Although many pigments that serve dim-light vision are very stable, even a molecule of vertebrate rod rhodopsin is thermally activated with a certain small probability, corresponding to a mean life time of ca. 3000 years at room temperature (Baylor et al 1980). Thermal activation will trigger the same transduction cascade as a photoisomerisation. After that, there will be no way of telling whether the quantal response has been initiated by light or by thermal energy. Such randomly occurring spontaneous photon-like events (“dark events”) will therefore cause a noise in the system that cannot even in principle be selectively suppressed by any signal processing, since it is identical to response to light. The signals from real light must cause a statistically significant increase to this background activity in order to be detected. The noise from “dark events” represents an ultimate limit to visual sensitivity (Aho et al 1988, Osorio and Vorobyev 2005).

These two main functional variables, spectral sensitivity and thermal stability, are interdependent for the following reason. Shifting the absorbance spectrum towards longer wavelengths for better performance in a “red” light environment implies lowering the activation energy of the pigment, and thus increasing the rate of thermal activations, the noise (Barlow 1957, Firsov and Govardovskii 1990, Ala-Laurila et al 2004a). In environments rich in long wavelengths, the advantage of increasing photon catch by red-shifting the absorbance spectrum will at some point be outbalanced or even reversed due to increased thermal noise. Evolutionary optimization of visual pigments works under these constraints, implying a compromise between the need to maximize photon catch and to minimize dark noise (Barlow 1957).

This is formalized in paper I of the present thesis, where performance is calculated as function of the pigment’s wavelength of peak absorbance \( \lambda_{\text{max}} \) in different aquatic environments. The signal \( S(\lambda_{\text{max}}) \) is directly proportional to photon catch within a certain time window, whereas the noise of a pigment is proportional to the Poisson variation of the number of thermal events \( N(\lambda_{\text{max}}) \) occurring within the same time window, i.e., noise = \( \sqrt{N(\lambda_{\text{max}})} \). The signal-to-
noise ratio of a pigment molecule in a
certain light environment is defined as
$SNR_{\text{pigment}} = \frac{S(\lambda_{\text{max}})}{\sqrt{N(\lambda_{\text{max}})}}$. Since
$N(\lambda_{\text{max}})$ is (assumed to be) a
monotonically increasing function,
$SNR_{\text{pigment}}$ will peak at shorter
wavelengths than $S$. At the very lowest
light levels, the visual (absolute)
threshold will depend on $SNR_{\text{pigment}}$, but
at somewhat higher light levels $N(\lambda_{\text{max}})$
becomes insignificant compared with
$S(\lambda_{\text{max}})$ and quantum catch alone
determines threshold.

The calculation of $S$ and $N$ in paper I
is simplified to consider the properties of
the pigment molecule alone. In real life,
$S$ depends on the optical density (OD) of
the photoreceptor cell along the axis of
light incidence and $N$ on the (generally
correlated) number of pigment molecules
in the cell. High thermal stability of the
pigment will allow a large number of
pigment molecules to be packed into the
photoreceptor cell without causing too
much noise. Thus the OD of rods
designed for vision in cool, dark
environments may be very high, in deep-
sea fishes even 1.0 around peak (Denton
1959). Quantum catch is then described
by the absorptance spectrum with a
comparatively broad and “flat” peak,
expressing that almost all incident
photons are captured over a certain
wavelength interval around the $\lambda_{\text{max}}$
of the visual pigment. In invertebrate
photoreceptors, the OD is presumably
smaller than in the best vertebrate rods
(Lythgoe 1979).

2.3.4 Molecular determinants of
functional properties

Chromophore

Chromophores in solution have
maximum absorbance in the near UV:
retinal has $\lambda_{\text{max}}$ close to 380 nm (in
solution ethanol; Knowles and Dartnall
1977) and 3, 4-dehydroretinal has a $\lambda_{\text{max}}$
at about 400 nm. Opsins themselves
absorb maximally in the far UV below
300 nm. However, these absorption
properties are not by themselves
significant in the visual process. When
retinal binds to opsin and the
chromophoric group forms; the
absorption spectrum is shifted into the
‘visible’ spectral region. In dim light
pigments, rhodopsins, the spectral shift
of retinal based pigments is
approximately 100-130 nm ($\lambda_{\text{max}}$
of RH1
$\approx$ 480-510 nm). The electrostatic
interactions between the opsin and the
chromophore determine the shape of the
absorbance spectrum and the exact
spectral location of $\lambda_{\text{max}}$. For any given
opsin, fishes as well as mysids may form
two pigments with different $\lambda_{\text{max}}$,
utilizing either of the two forms of
retinal, resulting in either a rhodopsin or
a “porphyropsin”. The additional double
bond in the 3, 4-dehydroretinal is
reflected in the generally longer $\lambda_{\text{max}}$
of the porphyropsin in a pigment pair, but
the effect depends on the location of $\lambda_{\text{max}}$:
the longer the $\lambda_{\text{max}}$ of the rhodopsin, the
greater is the long-wave displacement of
the porphyropsin. Several formulae for
relating the $\lambda_{\text{max}}$ of A2 and A1 pigments
has been proposed (Dartnall and
Lythgoe, 1965, Knowles and Dartnall
The wavelength difference between pigment pairs goes to zero at approx. 460 nm. For rod pigments with vitamin A1 as chromophore, the highest known $\lambda_{\text{max}}$ value is 584 nm (Britt et al. 2001). The $\lambda_{\text{max}}$ values of cone pigments may be even higher (approx. 630-635 nm; Kleinschmidt and Hárosi 1992, Bowmaker 1990, 1995, Bowmaker et al. 1991).

**Amino acid sequence**


Structural differences of the opsin chain underlie variations in its interaction with retinal. Principally, the smaller the energy difference between the ground state and the first excited state of the molecule, the longer is the wavelength of maximum absorbance. Substitutions of differently charged amino acids or amino acids with different polar properties may affect this energy difference. Basically, all amino acids have an influence on the structure and the formation of the opsin protein – even the C-terminus of the chain modulates it (Yokoyama et al. 2007) – but especially those amino acids that lie on the inner surfaces of the membrane helices and in close proximity to retinal have major roles in spectral tuning of the pigment (Bowmaker 1995).

There are many residues conserved in all visual pigments (Yokoyama 2002). They are primarily needed for maintaining the accurate structure and function of the pigment molecule. The residues that are in contact to the Schiff base are crucial; in all opsins this site is central to the light activation (Yan et al. 2003). All vertebrate visual pigments have lysine at position 296 (invertebrates: 305), and a negative counter ion, glutamic acid at residue 113 (in rhabdomeric opsins: 181; Lamb et al. 2007). The counter ion is a negatively charged amino acid that helps to stabilize the protonated Schiff base. The transmembrane region of the rhodopsin is stabilized by a number of interhelical hydrogen bonds and hydrophopic interactions and most of them are highly conserved in all GPCRs. The pair of
cysteines that form a disulfide bond, C110-C187 (squid, invertebrate: C108-C186, Murakami and Kouyama 2008, Shimamura et al 2008) and the sequence at the cytoplasmic surface of the pigment at position 134 (D/E), 135 (R), 136 (Aromatic Y, W, or F) are also conserved. This triad seems to be crucial in G-protein activation (Ebrey and Koutalos 2001). Two amino acid sites, 122 and 189 are important in distinguishing rhodopsin from cone opsins (Kuwayama et al 2002, 2005). The rhodopsins of jawed vertebrates have a Glu at position 122 and an Ile at position 189 (Kuwayama et al 2005). In invertebrates and in rhabdomeric opsins these residues vary (Lamb et al 2007).

2.4 Visual pigment adaptation: matching spectral sensitivity to the environment

2.4.1 Light environment and the challenge of dim light

The sun is the strongest source of light in the daylight hours. Before entering the surface of the earth, sunlight passes through the atmosphere and much of its light energy is absorbed and scattered by water vapour and the ozone layer. The scattered part of the light is partly deflected back into space, while the remainder reaches the ground as sky radiation. At the surface, the radiation from the sun is restricted to a spectral band from 300 nm to 1100 nm. The spectral distribution of sunlight and moonlight resembles each other, but sunlight is some 6 log units stronger than moonlight. Starlight is less bright than the full moonlight by an additional 3 log units (Munz and McFarland 1973). The special conditions of different microhabitats on the ground and under water are influenced by several other factors such as altitude, cloud cover, solar elevation, vegetation, water quality, etc. (Loew and McFarland 1990).

Animals have adapted to diverse photic environments by modifying their visual systems. One important aspect of this is the adaptation of the light-catching molecule, the visual pigment. There is a strong association between the types of visual pigments animals have and the environment they live in. This leads to the question: what are the particular functions for which different pigments are “adapted”? In scotopic vision, the problem is straightforward in principle, as rods are typically of a single kind and have the simple task to offer high visual sensitivity and achromatic contrast sensitivity in dim light. Rods have to use the prevailing light as well as possible (Lythgoe 1979). In aquatic environments the vision of most fish and small invertebrate species is challenged by the sparseness of photons in deep waters during at least part of their life cycle. They may use three main strategies to adapt to diverse photic environments: (1) changing the ratio of A1/A2 in chromophore (physiology); (2) mutation of opsin genes resulting in changes to the amino acid sequence of the opsin protein (evolution); (3) additionally some fish species are able to change the expression patterns of opsin genes.
2.4.2 The underwater light environment

The basic principles of light transmission are equal in air and water. The major difference is that absorption and scattering are more pronounced in the underwater light environment than in the air. The spectral characteristics of natural waters from oceans to freshwater have been carefully reviewed (e.g. Jerlov 1976). A brief summary about basic properties of underwater light environments is given here.

Water acts as a monochromator (Tyler 1959), absorbing both long and short-wave light. The maximum transmission (i.e., minimum attenuation coefficient) of pure water is in the region of 460-475 nm in the blue end of the spectrum. The wavelength-selective filtering of light is the reason why in the deep the spectral distribution of light is blue-dominated (Figure 5). In clear oceans, the attenuation of light is quite low. The dissolved salts in ocean water make virtually no difference to the absorption of visible light (Clarke and James 1939, Loew and Lythgoe 1978). The limit of photopic vision is reached at a depth of 300-500 nm (Bowmaker 1995). Theoretically, scotopic vision is possible as deep as 1000 m in the clearest oceans (Denton 1990). In coastal waters the amount of suspended particles will be higher and the limit for photic vision is reached at a depth of 30-50 m or even earlier (Bowmaker 1995). Fresh water usually contains even more suspended particles than coastal waters and therefore the limit for photopic vision may be at only a few meters. However, there are also relatively clear freshwater environments like Lake Baikal in Siberia and Crater Lake in Oregon, USA.

The water types are generally categorized according to the spectral transmittance of downward irradiance. Jerlov (1976) has established this classification, which applies to ten categories from open seas and coastal waters to brackish (e.g., Baltic) water. In all water types, the spectral irradiance will be very broad, from the near ultraviolet to far red, near the surface and in the uppermost few meters (if water is clear) (Figure 5). In the deeper parts, the spectral transmission of the water becomes dominant in each water type and the bandwidth of the light spectrum narrows (Lythgoe 1968, Dartnall 1975). The light attenuation is caused by the combined absorption and scattering properties of everything in the water column, including the water itself. Coastal, brackish and fresh waters are rather turbid containing variable amounts of dissolved organic matter (DOM). The blue colour of pure water does not change, but the amounts of chlorophyll and decay products are very variable depending upon the time of year, water temperature, the presence of nutrients in the water and the productivity (Loew and Lythgoe 1978).

The scattered light contributes to the underwater light environment and it is coloured partly by the absorption of the water and partly by Rayleigh scattering (Lythgoe 1979). The scattering of light affects the visibility of the objects: the outline of the objects may become less sharp. The amount of polarization is also reduced by the scattering and this
Fig. 5 Photic environment and the visual pigments of human, zebrafish and coelecanth. There is a strong correlation between the types of visual pigments animals possess and the light environments they live in. Humans have four different visual pigments, one rod and three cone pigments, covering a $\lambda_{\text{max}}$ range approx. from 420 nm to 564 nm. Near the surface of the earth or sea the light spectrum is wide: accordingly, zebrafish possess in addition to the rod pigment eight different cone pigments (Chinen et al 2003). In the deep sea the light spectrum is narrow: accordingly, coelacanths have only one cone pigment and a rod pigment (Yokoyama et al 1999). Due to the wavelength selective filtering by water, the light becomes almost monochromatic blue in the deep. The figure is reproduced with permission from Dr. S. Yokoyama; original figure is from Yokoyama (2008).

The concept of “optimality” of visual pigments is frequently highlighted in the context of the ecology of vision. There is now some consensus that a dim-light visual pigment should be judged by at least two major criteria. It ought to be tuned to coincide with the ambient light spectrum to capture as many photons as possible; and it ought to be as stable as possible, so the signal is not disturbed by excessive noise (degrading the SNR). This means that sometimes the maximal spectral match with the environmental light is not optimal, if this is associated...
with noise that overcomes the real signal. A third criterion, contrast detection, is usually more relevant in photopic conditions and when the animal possesses at least two visual pigments. The optimization of contrast detection is a more complex problem, even when it is not related to true colour vision. Colour vision poses additional theoretical questions on optimal numbers and relative spectral positions of different receptors (pigments), and the solutions will depend strongly on specific lifestyles. In the following I shall briefly review the different “hypotheses” that have (historically) been put forward about principles that guide the adaptation of visual pigments, with particular emphasis on dim-light vision.

**Sensitivity hypothesis**

According to the sensitivity hypothesis optimal visual pigments of the eye should have $\lambda_{\text{max}}$ values spectrally located to match the spectral composition of the environmental light, i.e., to maximise photon catch. It is necessary to sample enough photons to make reliable judgements about the image on the retina. In the deep sea with very little light narrowly distributed around 470-480 nm, rod pigments of fishes have $\lambda_{\text{max}}$ values roughly coinciding with the spectral maximum of the downwelling light, and are thus apparently well-adapted according to the sensitivity hypothesis (Lythgoe 1979, Crescitelli et al. 1985, Partridge 1988, Yokoyama and Tada 2000) (Figure 5). Other deep-sea animals like cephalopods (Kito et al. 1992), and crustaceans (Frank and Case 1988) have also shifted their spectral sensitivity towards the blue end of the spectra. The optimality of the visual pigment can be easily evaluated from the calculated relative quantum catch (QC), obtained by multiplying (convolving) the ambient light spectrum with the absorbance spectrum of the visual pigment. When judging the fraction of photons arriving at the cornea that are absorbed and transduced, the variable optical properties of eyes including ocular filters must also be taken into account. In different animal species only 25-59 % of corneal photons lead to the production of an electrical single-photon response (Warrant 2004). The sensitivity hypothesis is most relevant in dim light conditions where the selective adaptation pressure to tune the visual pigment is high. However, it does not explain why fishes that live in green coastal water and in most fresh waters have visual pigments that are not sufficiently red-shifted to give maximum quantum catch. Therefore another hypothesis is needed.

**Noise hypothesis**

The noise hypothesis is based on the fact explained above (Section 2.3.3) that the visual pigment molecules will necessarily be activated by thermal energy with some probability and will then produce a dark noise signal. This type of thermal noise in the cell consists of randomly occurring discrete events indistinguishable from the responses to single photoisomerizations. According to theory first presented by Barlow (1957), the rate of thermal isomerisation of a visual pigment depends on its
wavelength of maximum absorbance ($\lambda_{\text{max}}$). The more red-sensitive pigments have lower energy barrier for excitation than the more blue-sensitive ones and therefore higher probability that the energy barrier may be overcome by thermal energy alone. It has been shown that the frequency of thermally induced noise events does increase with increasing $\lambda_{\text{max}}$ (Firsov and Govardovskii 1990, Ala-Laurila et al 2004b) and that A2 based pigments are noisier than the A1 based pigments (Donner et al 1990, Ala-Laurila et al 2007). Thus the visual pigment giving the highest signal-to-noise ratio and thus allowing the highest absolute visual sensitivity may be blue-shifted from the $\lambda_{\text{max}}$ that would provide the highest quantum catch.

Another case where spectral sensitivity changes in a way best consistent with the noise hypothesis is related to the A1/A2 system in fishes. In the summer the fishes that possess the A1/A2 system increase the proportion of the A1 pigment in their retinas. This phenomenon is inconsistent with the spectral change in the environmental light, which is often red-shifted. One possible reason is that decreasing the proportion of “noisy” A2 pigment brings a decrease in thermal noise that more than compensates for the poorer spectral fit to the environmental light.

The noise hypothesis recognize a universal limitation of biological systems: the thermal movements of molecules and the noise originated of it. In paper I, the optimal locations of visual pigments in Baltic Sea fishes have been calculated on one hand under the sensitivity hypothesis and on the other hand under the noise hypothesis, using reasonable assumptions about the relation between $\lambda_{\text{max}}$ and pigment noise. While setting an ultimate limit to the sensitivity of dark-adapted vision, pigment noise is not seriously detrimental to visual sensitivity in bright light, where rates of photoisomerizations exceed rates of thermal activations by orders of magnitude. However, in hue discrimination pigment noise may still be a limiting factor (Vorobyev and Osorio 1998).

**Contrast hypothesis**

A third idea, the contrast hypothesis, has been proposed (Lythgoe 1972, 1979) to explain why the $\lambda_{\text{max}}$ values of visual pigments of many freshwater and coastal fishes do not match the greatest light flux of the ambient illumination. The contrast hypothesis is based on the realization that visual tasks are usually problems of contrast detection. Vision is designed to see objects, for example, predators and prey or a suitable mate, rather than to see light. In underwater light environments objects often have low contrast, defined as the proportional difference between the target radiance and the background radiance (Duntley 1963). Hence, a pigment slightly offset from the background spectrum may be beneficial in some situations.

Ideally, the contrast hypothesis requires at least two visual pigments, one of them matched to the wavelength of maximum background radiance and one offset from that wavelength. The contrast hypothesis does not apply to the rod
pigments or the *Mysis* pigments considered in this thesis, since these function as sole pigments in dim light conditions where every quantum is important. If the absorbance maximum of the visual pigment is located off the spectrum of environmental light, the numbers of photons absorbed will inevitably be reduced. The contrast hypothesis is more relevant in conditions when more light is available to uphold several photoreceptor classes and especially in cones. However, colour vision is not necessarily related to the contrast hypothesis, although possession of at least two visual pigments is the necessary precondition for colour vision (Lythgoe 1979). Lythgoe and Partridge (1991) have developed a colour vision model based on the contrast hypothesis, which predicts the optimal wavelength regions of the visual pigments in green coastal waters. Marshall and his colleagues (2003) have further modified this model using Hawaiian reef fishes. The double cones of some teleosts seem to match the underwater background space light much better than the rods (Lythgoe 1979, 1984, Lythgoe et al 1994, Bowmaker et al 1994). It has been proposed that they could have been specialized for detection of distant and dark objects and other cones are specialized for chromatic discrimination (Bowmaker et al 1994).

**Colour vision**

A ‘fourth’ class of theories about optimal sets of spectrally different pigments applies to wavelength discrimination and colour vision, which is useful mainly in photopic conditions where a broad spectrum of light is available. The animals inhabiting these photic conditions should have a variety of different photoreceptors and visual pigments (Figure 5: zebra fish). The question of optimization of these visual pigments and photoreceptors is more complex than under the previously discussed hypotheses, which deals only with detection of light or discrimination of contrast. The outcome and benefit of several visual pigments and photoreceptors is presumably colour vision as in many teleost fishes (Loew and Lythgoe 1978, Lythgoe 1979, Bowmaker et al 1994). In these conditions the optimisation of the number of different photoreceptors is one of the essential elements. The smallest signals that can be usefully coded depend upon the level of noise. The discriminability of natural spectra can be increased by increasing the spectral separation of photoreceptors or by sharpening the absorbance spectra by means of oil droplets, but what is really efficient depends on the biological task. For example, the uniformly spaced pigments of honeybees are optimal for discriminating flower colours (Chittka and Menzel 1992, Vorobyev and Menzel 1999) whereas detection of fruit against leaves may favour the closely spaced L and M pigments of trichromatic primates (Osorio and Vorobyev 1996, Sumner and Mollon 2000).
2.4.4 Mechanisms of spectral adaptation

In the course of evolution the primary selection pressure has been set by the spectral range and the intensity of daylight. An example of fundamental adaptation to different light intensities is that nocturnal vertebrates have rod-dominated retinas whereas diurnal species have cone-rich retinas. Further, visual sensitivity can be adapted by spectrally tuning the sensitivity of the visual pigments and/or varying the number of photoreceptor classes.

Fishes have several ways of tuning the spectral sensitivity of vision:

1. Many freshwater fishes have the previously mentioned capacity to change the proportions of chromophores A1 and A2 depending on the season (light level, temperature; Dartnall et al 1961, Allen and McFarland 1972) the developmental state (Bridges 1972) or the physiological state of the animal. Generally, visual pigments based on A2 have long wavelength-shifted absorbance-maximum and the spectra is broader compared to those of A1-derived pigments. However, the disadvantage is that the A2 pigments are more susceptible to noise than the corresponding A1 pigments.

2. On an evolutionary time scale, spectral sensitivity is tuned by mutations resulting in amino acid substitutions in the opsin protein (Yokoyama 2002, Takahashi and Ebrey 2003, Yokoyama et al 2008). The spectral absorbance of the visual pigment is determined by the interaction between the chromophore and specific amino acids of the opsin. The $\lambda_{\text{max}}$ shift achieved with a single substitution varies from 0 nm to 75 nm (Shi et al 2001). The effect depends mainly on the location of the substitution in the opsin or the chemical properties of the substituted amino acid (Hunt et al 2001). The mutations can occur only in a limited number of sites within the opsin without producing a non-functional pigment or a pigment without prospective spectral shift (Hunt et al 2001). Understanding spectral tuning is complicated because there are multiple ways to produce similar absorption spectra (Yokoyama 2008).

3. A third method is by varying the sets of opsins/photoreceptors that are expressed among a larger number of pre-existing genes. Some African cichlid species use differential set of cone opsin expression to modulate spectral sensitivity (Carleton and Kocher 2001, Parry et al 2005). Individual species express a unique subset of the seven possible cone opsin genes. During the ontogenetic development some of the genes may be up- or down-regulated, so that the larval and adult retinas have different sets of opsins/photoreceptors. Age-related loss, or reduction, of ultraviolet photoreception is reported in many species of fish, including salmonids (Bowmaker and Kunz 1987, Hawryshyn et al 1989, Whitmore and Bowmaker 1989, Loew and Wahl 1991).

4. There are also other ways of spectral tuning not dependent on the visual pigment, for instance the use of ocular filters (Douglas and Marshall 1999, Cronin et al 2001, Cheroske et al 2003), screening pigments (Cronin and Marshall 1989), and oil droplets (Douglas and Marshall 1999).
A1-A2 chromophore shifts

The A1/A2 ratio is regulated by chromophore supplied by the pigment epithelium (Bridges 1972) and the Müller cells (Wang et al 2009). Photoreceptors of the same type in close proximity therefore have the same ratio of A1/A2 pigments (Loew and Dartnall 1976), although the ratio can vary throughout the retina (Muntz and Northmore 1971, Reuter et al 1971) and e.g. between rods and cones (our unpublished observations).

The proportion of A1 and A2 can be regulated in response to environmental factors in several fish species (seasonal changes: temperature and photoperiod; Dartnall et al 1961, Beatty 1969, Allen and McFarland 1972, Allen et al 1982, Ueno et al 2005), amphibians (Makino et al 1983), and invertebrates (Suzuki et al 1984). These changes are apparently due to the regulation of the chromophore synthesis enzymes. The alteration of the A1/A2 pigments is also related to the developmental stage of the animal. In some teleost species (e.g. rudd, Scardinius erythrophthalmus) the A1/A2 ratio is more constant in older individuals than in younger ones (Bridges 1972). Moreover, the ratio of rhodopsin/porphyrpsin can vary across the retina (Bridges and Yoshikami 1970, Kusmic and Gualtieri 2000) or among individuals (Whitmore and Bowmaker 1989).

The $\lambda_{\text{max}}$ of the A1 pigments varies from approximately 350 nm to 584 nm and in A2 pigments from approximately 510 to 635 nm (Dartnall and Lythgoe 1965, Kleinschmidt and Hárosi 1992, Archer 1999, Britt et al 2001). In marine species A1 predominates whereas freshwater species more often utilize A2 pigment. Mixtures occur in many species (catadromous, anadromous species), which migrate between marine and more freshwater environments with different spectral compositions of light (Wald 1941, Carlisle and Denton 1959, Bridges 1972). The migratory species utilize the A2 based pigments in freshwater basin and the A1 based pigments in marine conditions. The change is presumably regulated by the hormones thyroxine and prolactine (Munz and Beatty 1965, Cristy 1974, Kusmic and Gualteri 2000).

Amino acid substitutions

Over the past quarter century (Ovchinnikov 1982, Hargrave et al 1983, Nathans and Hogness 1983, 1984) the rapid accumulation of nucleotide sequences of diverse opsin genes in vertebrate and invertebrate species has increased the understanding about molecular interactions of the pigment. At least 470 visual opsin genes from approximately 180 vertebrate species have now been characterized (Yokoyama 2008). For 126 of these genes the complete nucleotide sequences have also been related to the $\lambda_{\text{max}}$ value of the corresponding visual pigment using in vitro assays (Yokoyama et al 2008). This has broadened the understanding of how pigments are spectrally tuned and the constraints that may be acting on this tuning. Spectral tuning has been most intensively studied in vertebrates, where site directed mutagenesis and in vitro
expression systems have allowed researchers to measure the effects of single amino acid changes on $\lambda_{\text{max}}$ (Yokoyama 2002). Studies with various mutations introduced into the engineered ancestral pigments show that a total of nine amino acid replacements (D83N, Y96V, Y102F, E122Q, E122I, P194R, N195A, F261Y and A292S) explain the $\lambda_{\text{max}}$ values of most ancestral and contemporary RH1 pigments (Yokoyama 2008).

The three-dimensional model of the opsin (Palczewski et al 2000) has helped to identify candidate spectral tuning sites. Critical amino acid changes that cause considerable $\lambda_{\text{max}}$ shifts individually or synergistically are localized to a total of 30 residues, most of which are located near the N terminus of the TM segments (Yokoyama 2008). Three major functional requirements on the rod opsin sequence (quantum catch, thermal stability, and molecular restrictions related to structure and enzymatic function) may explain why there is a relatively limited number of sites that can be allowed to change to give the required spectral shifts (Hunt et al 2001). Potential tuning sites are generally identified as those that are in the vicinity of/or oriented toward the chromophore and make up the chromophore binding pocket. The substitution is nonconservative, i.e. between polar and nonpolar or charged and noncharged amino acids (Hunt et al 2001, Takahashi and Ebrey 2003, Yokoyama and Tada 2003, Spady et al 2005). However, there are some exceptions to these rules: e.g., residues 102, 194, 195 in RH1 pigments and 197 M/LWS pigments (bovine: site181) are located in the luminal face, outside the transmembrane segments (Yokoyama 2008). The retinal-opsin interactions extend well beyond the retinal binding pocket. The C-terminus of the rhodopsin modulates $\lambda_{\text{max}}$ by interacting mainly with the TM VII (Yokoyama et al 2007). In addition, the residues 194 and 195 are located approximately 20 Å away from the chromophore. The mechanism of long distance amino acid interactions is not fully understood at the moment (Yokoyama 2008).

The $\lambda_{\text{max}}$ of a visual pigment depends on at least two factors. (1) The strength of the electrostatic interaction between the Glu113 counterion and the protonated Schiff base. Substitutions that increase the strength of this interaction and stabilise the ground state will result in a shortwave shift, whereas those that reduce it will generate a shift towards longer wavelengths. (2) Photoexcitation of the chromophore induces an increase in $\pi$ electron delocalization. All interactions that will ease the delocalization of the $\pi$ electrons will result in a long wave shift in the absorbance spectrum and those interactions that prevent the delocalization will result in a shortwave shift (Nathans 1990, Hunt et al 2001).

The distance between the protonated Schiff base and its negative counterion is essential for the determination of the absorbance maximum of the pigment. If the distance is increased, the spectrum will shift towards longer wavelengths (Blatz et al 1972). The substitution E113Q in TM3 will blue-shift the spectra by 120 nm (Sakmar et al 1989). The side chains of the amino acids can cause steric
interactions (Han et al 1996) and bind ions that will alter the absorbance maximum of the visual pigment (Cl’ binding in cones, Wang et al 1993). The dipolar and electrostatic environment of the retinal as well as the solvent cavities which are occupied by water will strongly influence the spectrum (Teller et al 2003). Non-polar residues may also play a role by sterically constraining the torsion angles of the retinal.

Similar functional changes can be achieved by different amino acid replacements. For example D83N/A292S, P194R/N195A/A292S and E 122Q all decrease the \( \lambda_{\text{max}} \) by 14-20 nm (Yokoyama 2008). Additionally, mutagenesis experiments have revealed that the functional change of the same amino acid replacement is highly dependent on the background amino acids of the opsin. For example, G90S in a RH1 pigment decreases \( \lambda_{\text{max}} \) by 13 nm, but the reverse change, S90G in a SWS1 pigment decreases \( \lambda_{\text{max}} \) by 7 nm (Yokoyama 2008). This demonstrates that synergistic interactions occur between different amino acid residues. The spectral tuning is further complicated when considering the forward and reverse mutations. Even if the amino acid changes causes spectral shifts to opposite directions, the magnitudes of \( \lambda_{\text{max}} \) shifts can differ significantly due to different amino acid background. For example pairs F86Y and Y86F, A269T and T269A and A292S and S292A shift \( \lambda_{\text{max}} \) values to opposite directions, but the difference in the magnitudes is more than 10 nm (Yokoyama 2008).

**Altered gene expression and other strategies**

Genes may be present but not expressed or only expressed at particular stages of an animal's life. The deep-sea opsin of the eel (Carlisle and Denton 1959, Beatty 1984) and the blue pigment of the pollack (Shand et al 1988) may be examples of this. Black bream have six different cone opsin genes which are differentially expressed in the retina during the ontogeny (Shand et al 2008). Similar changes in gene expression is also reported in Nile tilapia, expression of the SWS1 gene is related to larval and juvenile stages (Spady et al 2006). African cichlid fishes (Carleton and Kocher 2001) have seven distinct cone opsin genes; of which each species utilize the subset of five opsin genes. In the Pacific salmon and rainbow trout (Cheng and Novales Flamarique, 2004, 2007), the switch from expressing SWS1 to SWS2 has been shown to happen in individual single cones during development, whereas in the zebrafish new opsins are expressed in newly differentiated photoreceptors as the retina progressively grows (Takechi and Kawamura 2005). Differential gene expression associated with ontogenetic changes and metamorphic transitions is a widely used mechanism to tune the visual system (Shand et al 2002, Mader and Cameron 2004, Takechi and Kawamura 2005, Shand et al 2008, Temple et al. 2008).

In addition to the visual-pigment-related mechanisms considered above (chromophore exchange, amino acid substitutions and altered gene
expression) the spectral sensitivity of vision may be modified by several kinds of spectrally selective filters interposed in the light path in front of the visual pigment, e.g. corneal coloration or oil droplets such as found in the ellipsoid of cones of many vertebrates (see e.g. Johnston and Hudson 1976, Lythgoe 1979). The oil droplets in cones most often serve to improve wavelength discrimination by narrowing spectra and increasing the classes of spectrally different cones. In other cases, filters clearly have a protective role, especially against short-wavelength light. In paper IV, ocular screening pigments are shown to change strongly the spectral sensitivity of *Mysis relicta* compared with the absorbance spectrum of the visual pigment.

### 2.5 Selection of model species

The selection of animal models in the present work was guided by two main considerations. (1) The objective was to study single dim-light pigments in spectrally differentiated aquatic environments. Hence, rod pigments of many fish species, as well as aquatic invertebrates with a single visual pigment, dwelling in deep waters at least part of the time, were suitable. (2) To provide information on changes that have occurred on short to intermediate evolutionary time scales, the animal models should comprise genetically isolated populations, subspecies and species that have inhabited spectrally different environments for known times covering a range from ca. $10^4$ to $10^6$ years. The (postglacial) Baltic Sea and Fennoscandian lakes offer excellent models for the shortest time scales. Thus the choice was to study Baltic fishes and the opossum shrimp (*Mysis relicta*). Among the fishes, the sand goby (*Pomatoschistus minutus*) and closely related gobies were chosen for more detailed study. Deeper history was provided by broader comparisons of several sand goby populations from the coasts of Europe and related goby species, as well as spectral differentiation between sibling species of the *M. relicta* species group.

**The sand goby (Pomatoschistus minutus): ecology and phylogeny**

The sand goby is a small, short-lived, ecologically important fish that is very abundant along the Atlantic coasts of Western Europe (Healey 1971). Different subspecies occur in the Black Sea, the Mediterranean Sea and the north-eastern Atlantic Ocean from the south of Spain to northern Norway (Tromsø) and in most parts of the Baltic Sea (Miller 1986). The sand goby performs seasonal migrations, spawning in very shallow waters in spring and going down to at least 40 m depth in autumn (Ehrenberg *et al* 2005). It swims significantly more in darkness than in light and buries in the sediment during day (Ehrenberg and Ejdung 2008).

The phylogenetic history of sand goby has been much studied recently (Stefanni and Thorley 2003, Gysels *et al* 2004, Huyse *et al* 2004). The genetic
differentiation appears to be rather weak in the Atlantic and the Baltic basins, whereas the larger genetic distance between the Venetian and all the other P. minutus indicates allopatric speciation in the Northern Adriatic Sea. Adriatic sand goby should now be considered as a distinct species (Stefanni and Thorley 2003, Huyse et al 2004). The exact timing of the speciation between the Atlantic and the Adriatic group is estimated to be in the late Pliocene or early Pleistocene (≈ 2.5-1.7 mya BP). The invasion to the Baltic Sea occurred quite recently, approximately 8000 years ago (Gysels et al 2004). The sibling species studied in the present thesis, the marbled goby (P. marmoratus) and the common goby (P. microps), are more closely related to each other than to the sand goby (Huyse et al 2004). The last common ancestor of P. minutus, P. marmoratus, and P. microps probably occupied in the middle of the Pliocene (≈ 3.5 mya BP; Huyse et al 2004).

The opossum shrimp (Mysis relicta): ecology and phylogeny

The crustacean order Mysida consists of over 1000 described species which inhabit a variety of environments with different spectral composition of light. These species are found across all latitudes throughout subterranean, fresh, brackish, coastal, oceanic, and surface to deep sea (>7000m) waters. The species of the M. relicta group studied in this thesis are found in deep oligotrophic and mesotrophic lakes in the northern regions of North America, as well as in the seas of the northern regions of Eurasia. Mysids are comparatively small crustaceans (5-50 mm), which play an important role in aquatic ecosystems both as predators and prey. They avoid light, remaining in the deeper parts of the water during the day and moving up to the epilimnion at night (Beeton and Bowers 1982). The light intensity together with the temperature seems to be the main factor in these diel migrations (Gal et al 1998).

The M. relicta species group consists of four sibling species, that until recently were considered to be subspecies of a single species M. relicta: (1) The European stenohaline M. relicta, sensu stricto, (2) the European euryhaline M. salemaai, 3) the subarctic M. segerstralei and 4) the North American freshwater M. diluviana (Väinölä 1986, Väinölä et al 1994, 2002, Audzijonytè and Väinölä 2005, Dooh et al 2006). The species found in the Baltic Sea, M. salemaai and M. relicta, appear to have diverged approx. 2 mya BP in the early Pleistocene; whereas, the isolation of “Lake” and “Sea” populations of M. relicta is much more recent, connected with the postglacial separation of Finnish and Swedish lakes from the Baltic Sea.

2.6 The Baltic Sea and postglacial Fennoscandian lakes as an evolutionary laboratory: freshwater and marine species

The Baltic Sea is the largest brackish water body / inland sea in the world (Tikkanen and Oksanen 2002). It is also
one of the youngest seas on the Earth. The water connection to the North Sea is through the narrow Danish straits and Kattegat between Denmark and Sweden. The environmental conditions of the Baltic Sea are defined by the fresh water input from rivers and by the limited inflow of more saline water from the North Sea. Without the small influx of saline water through the Danish straits, the Baltic Sea would have been transformed become a fresh water lake long ago. The mean depth is 55 m, but the deepest parts reach down to 459 m. Salinity is much lower than in the ocean, varying from 0.1 % in the north to 0.6-0.8 % in the center. Below 40-70 m, salinity can be as high as 1.5-2.0 % (Tikkanen and Oksanen 2002).

Over the last million years northern Europe, including the Baltic basin was repeatedly covered by ice sheets. During its history, the sea has undergone several distinct evolutionary phases. Periodically, it has been isolated from the ocean, and then it has been a fresh-water basin which subsequently has turned into a typical intra-continental sea. About 10000 years ago, the Baltic Ice Lake was connected to the ocean through central Sweden. The Strait in central Sweden closed approximately 9500 years ago and the so-called Yoldia Sea again turned into a fresh-water lake (Ancylus Lake). The connection to the Atlantic Sea through the Danish Straits opened approximately 8500 years ago resulting in the warm Littorina Sea. Finally, approximately 4000 years ago, uplifting movements in the Danish Straits resulted in a reduction of water exchange with the North Sea, and the brackish Baltic Sea was formed (Tikkanen and Oksanen 2002).

The rapid abiotic changes during its geologically young history have shaped the fish community of the Baltic Sea. Especially important have been the changes in salinity. The fish fauna in the Baltic Sea include marine species (e.g. cod, flounder and herring), anadromous (e.g. Atlantic salmon and Sea trout) and catadromous species (e.g. European eel) as well as fresh water species (e.g. pike and perch). Altogether approximately 100 fish species are abundant in the Baltic Sea; 35 of them are freshwater species. The fish species have immigrated by different routes at several occasions. However, the distribution pattern of the various species reflects their original habitat and salinity tolerance.

The origin of the Finnish lakes dates back to the deglaciation some 10000 years ago. The fragmented coastline and the lakes were formed as the edge of permanent ice retreated to the north. The land uplift separated fresh-water lakes from the Baltic Sea (Tikkanen and Oksanen 2002). The mean depth of Finnish lakes is only 7 meters although deeper lakes also exist. All fishes in the Baltic area have migrated there during the last 10000 years, but isolation has not been complete for all species, since there has been some gene flow from the North Sea.

The Baltic Sea offer unique possibilities for studying short-term evolution of aquatic species. It has been Nature’s own laboratory for testing their adaptation capabilities. Typical
modifications in the brackish-water sea are related to growth. Marine species tend to grow slower and to be generally dwarfed whereas freshwater species tend to grow bigger. Vision has had to cope with the shifting of the light irradiance spectrum towards longer wavelengths compared with the real marine conditions. On the other hand, many freshwater lakes are even further red-shifted, as humus and other organic matter strongly absorb shorter wavelengths.
3 Aims of the study

The general objective of this thesis was to study the scope, tempo and mechanisms of spectral adaptation of visual pigments on short to intermediate evolutionary time scales. Our choice of animal models was guided by two main considerations:

(1) The most basic task of visual pigments is to catch as many photons as possible. This function is particularly challenged in dim light conditions when only one visual pigment is working. Aquatic light environments offer “Nature’s own laboratories” for basic research on visual pigment properties, as naturally occurring spectral environments vary greatly between different geographical locations. Our first consideration was that there should be clearly definable selection pressures; therefore, we studied pigments that serve vision in very dim light in spectrally differentiated aquatic environments. From this point of view, rod pigments of many fish species, as well as the single visual pigment of many invertebrates would be suitable.

(2) Second, we needed populations, subspecies and species that had been genetically isolated in spectrally different habitats for known times covering a range from ca. $10^4$ to $10^6$ years. The (postglacial) Baltic Sea and Fennoscandian lakes offer excellent models for the shortest time scales. Thus we chose to study Baltic fishes, particularly the sand goby *Pomatoschistus minutus*, and *Mysis relicta* crustacean shrimps. Somewhat deeper history was provided by broader comparisons of several sand goby populations from the coasts of Europe and related goby species, as well as spectral differentiation between sibling species of the *Mysis relicta* group.

The more specific aims of the separate studies were as follows:

**Paper I**: The empirical purpose was to study whether spectral divergence of the rod pigment could be found in Baltic populations of fish species that had originated, on one hand, from the more short-wavelength-biased environment of the Atlantic Ocean, on the other hand from the generally more long-wavelength-biased environments of fresh-water lakes. The theoretical purpose was to define the different measures of good performance required by the “noise hypothesis” as opposed to the “sensitivity hypothesis”, given present knowledge of spectral and thermal properties of A1 and A2 visual pigments.

**Papers II and III**: The purpose of these studies was to characterize visual pigment adaptation phenotypically and genotypically at different levels within species and between species. The sand goby (*Pomatoschistus minutus*) was chosen as model for within-species evaluation of spectral tuning of the rod pigment and its relation to amino-acid substitutions in the opsin. Rod rhodopsins of other goby species were investigated as outgroups for comparison.

**Paper IV**: The purpose was to study possible adaptation of the absorbance spectrum of the visual pigment and the
spectral sensitivity of vision on a very short evolutionary time scale in an invertebrate with low mobility and short generation intervals: the opossum shrimp (*Mysis relicta*). Populations have been postglacially isolated in several Finnish lakes, some with deep brown water, as recently as 9000 years ago. Strongly differing spectral requirements combined with exact knowledge of isolation history makes this an attractive model. The sibling species *M. salemaai* and *M. diluviana* of the circumpolar *M. relicta* group were studied for comparison.
4 Materials and methods

4.1 Animals

A total of 26 teleost fish species and 3 opossum shrimp species from the *Mysis relicta* species group (Crustacea, Mysida) were included in this thesis. We obtained fish samples by netting from the Baltic Sea (22 spp.) along the coast of Finland: the south-west coast: Tvärminne Zoological Station, Hanko and the Archipelago Sea near Nagu; the south coast: Gulf of Finland at Kotka; Figure 6). Samples of freshwater fish populations (9 spp.) were from four Finnish lakes (Lake Vesijärvi, Lake Päijänne, Lake Tuusulanjärvi, and Lake Bromarv Pond (Framnäs), Figure 7). Marine specimens (4 spp.) were obtained from the Eastern Atlantic (flatfish from Raunefjord south of Bergen, Norway and herrings originating from the east coast of Scotland received as a gift from the Sea Life Aquarium, Helsinki), from the west coast of Sweden near Kristineberg (sand gobies), from the English Channel near Plymouth, England (sand gobies) and from the Adriatic Sea near Venice, Italy (sand gobies and marbled gobies). The opossum shrimps were from 8 different populations (Figure 6). Four Finnish populations, two from the Baltic Sea and two from freshwater lakes, represent *Mysis relicta*, sensu stricto. The sibling species *M. salemaai* and *M. diluviana* are represented by, respectively, two Baltic Sea populations and two populations from freshwater lakes in Idaho, USA.

After netting, fish were transferred in special water bags or tanks to the animal care facilities of the University of Helsinki. They were kept in aquaria with the salinity of their natural habitats at approx. 15 ºC and 12:12 h light/dark cycle, and supplied with appropriate food (for details see paper I and II). Some of the fish were frozen (-18 ºC) immediately after netting and stored frozen in darkness until measurements. We found no significant difference in $\lambda_{\text{max}}$ of cells from retinas that had been frozen and then thawed compared with fresh retinas of fish of the same species from the same habitat.

After capture, opossum shrimps were kept in total darkness in aerated aquariums, with water temperature of 3-8 ºC approximating their natural habitats. Especially some lake populations of *Mysis relicta* have highly light sensitive eyes and their eyes are easily damaged if exposed to excessive light (Lindström and Nilsson 1988, Lindström et al 1988). These animals were caught at night and care was taken not to expose them to stronger light at any time before measurements.

4.2 Methods and data analysis

4.2.1 Microspectrophotometry (MSP; I, II & IV)

Microspectrophotometry is a technique which enables direct measurements of the absorbance spectra from isolated
Fig. 6 Geographical locations and the spectral light environments of the animals studied in this thesis. The left panel illustrates the geographical locations of the fish and the Mysis habitats (except M. diluviana from North America). Squares with no filling represent M. relicta and coloured circles the different fish species. The colour codes correspond to the light spectrum of the same geographical location in the right panel. On the right are normalized photon flux spectra from marine/brackish and freshwater environments. The loci and the depths of measurement are: Lᵥ: Lake Vesijärvi (10 m; paper I), Lᵧ: Lake Tuusulanjärvi (4 m; paper I), Lₚ: Lake Pääjärvi (5 m; Lindström 2000), JI: (100 m; recalculated from Jerlov 1976) representing Adriatic Sea, JIII: (100 m; recalculated from Jerlov 1976) representing English channel and Northern Atlantic, B: Baltic Sea (20 m; Lindström 2000), Bₚ: Pojoviken of the Baltic Sea (10 m; Lindström 2000).

Photoreceptors. It has been widely used to record the absorbance spectra of visual pigments in situ in vertebrate and invertebrate photoreceptors. In practice, thin light beam with different wavelengths (350-790 nm) is focused on the anatomical structure harbouring the visual pigment (the outer segment (OS) of a vertebrate rod or a single rhabdom of a Mysis). Some of the light is absorbed by the visual pigment and the rest is captured by a photomultiplier tube. The idea is to determine the ratio of entering and emerging light and thus the fraction of light at each wavelength that is absorbed by the visual pigment.

For details, the reader is referred to the original papers (I, II, & IV). In brief, dark-adapted animals were decapitated and double-pithed. All manipulations were carried out under infrared light in
order to prevent bleaching of the visual pigments. Retinas were carefully isolated and a small sample was prepared immediately for measurements. Fishes (gobies, II) were individually marked and the bodies frozen separately in plastic bags for DNA extraction and opsin gene sequencing (III). For the MSP a small piece of tissue was dissected from the retina, teased apart and covered with a second coverslip. Absorbance spectra were then recorded with a single-beam, computer-controlled, fast wavelength-scanning microspectrophotometer built at the University of Helsinki (Govardovskii et al. 2000, Ala-Laurila et al. 2002). The basic design is described in Govardovskii and Zueva (2000). For further technical details, see Govardovskii et al. (2000) and Ala-Laurila et al. (2002).

The data were stored on the computer hard disk for later analysis. The details of the analysis can be found in Govardovskii et al. (2000). Raw spectra from single cells were averaged and normalized within each individual animal, and the resulting within-individual average was corrected for zero offset. Finally, the within-individual average spectrum was fitted with Govardovskii et al. (2000) templates. The width and shape of the spectrum depends on the chromophore, the A1 template being narrower and the A2 template broader. Therefore, the chromophore and the ratio of the chromophore mixture (A1/A2) were basically determined from the best-fitting template or sum of templates. Due to the standard shape of spectra, all essential information can be summarized in two parameters: $\lambda_{\text{max}}$ and the A1/A2 ratio (Govardovskii et al. 2000).

4.2.2 Electroretinogram (ERG) measurements of spectral sensitivity (IV)

Spectral sensitivity of Mysis shrimps was determined using electroretinogram (ERG) technique. In the ERG measurement the mass response of the eye to light of different wavelengths (472 – 777 nm) was measured directly by inserting the active electrode, a glass micropipette with ca.10 $\mu$m tip diameter, into a dark adapted eye close to the layer of rhabdons. The eyes were allowed to adapt in complete darkness for about 1 hour before starting the measurements, since the eyes are highly sensitive to long wavelengths (Lindström and Meyer-Rochow 1987). The wavelength 512 nm served as reference; response amplitude per incident photon was measured in the linear response range for 14 other wavelengths and related to this. For details, see Donner (1971), Lindström et al. (1988), Pahlberg et al. (2005), and paper IV. Over most of the spectral range, sensitivity measured in ERG experiments was strongly decreased by the absorption of intraocular filters (screening pigments) present in the ommatidia (Goldstein and Williams 1966, Goldsmith 1978). In Mysis eyes of isolated heads (as studied here), a screening pigments were apparently positioned as they would naturally be in light-adapted eyes, and only at very long wavelengths (> 700 nm) does electrophysiological sensitivity parallel visual-pigment absorbance (IV).
4.2.3 Opsin sequencing (III)

Opsin sequencing was based on determining the DNA sequence of the gene, which encodes the information on the amino acid sequence. In brief, genomic DNA was extracted and purified from a piece of muscle tissue of individually identified, frozen gobies (*P. minutus*, *P. microps*, and *P. marmoratus*). The work was carried out according to the instructions of the manufacturer (Dneasy™ Tissue Kit, QIAGEN, Hilden, Germany) and the genomic DNA was stored at -20°C until use. The primer sets were designed according to the sand goby rhodopsin sequence published by Archer *et al* (1992; EMBL/GenBank X62405) to amplify a rod opsin gene in one or two piece(s) in PCR (polymerase chain reaction). Additional primers were generated according to the obtained opsin sequences. The sequencing reaction was done using BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA) and an ABI3100 capillary sequencer system (Applied Biosystems, Foster City, CA, USA). The obtained sequences were assembled and edited using the GAP4 program from the Staden Package (Bonfield *et al* 1995). For more information see Materials and methods section of paper III.
5 Results

Differences in spectral properties of visual pigments between populations isolated in spectrally different environments (I, II & IV)

Absorbance spectra were recorded by microspectrophotometry (MSP) in rods and some cone types from a total of 26 fish species belonging to 12 families from 39 different geographical and spectral locations (I & II). Additionally, in paper IV absorbance and sensitivity spectra were recorded by MSP from eight populations of opossum shrimp and also by ERG from six populations of opossum shrimp from different light environments. Four of these were Finnish 
*Mysis relicta* populations, two from the Baltic Sea and two from freshwater lakes. The sibling species *M. salemaai* and *M. diluviana* were represented by two Baltic Sea and two freshwater lake (Idaho, USA) populations, respectively.

Each within-individual average spectrum was fitted with a Govardovskii et al (2000) template (see Materials and Methods) to determine the parameter $\lambda_{\text{max}}$ as well as the apparent chromophore of the pigment (A1, A2 or the A1/A2 ratio of a mixture). Knowing the chromophore is essential for the interpretation of the results, as a spectral shift may be achieved either by substitutions in the amino acid chain of the opsin occurring on an evolutionary time scale or a chromophore exchange occurring on a physiological time scale. All of the marine fish species used pure A1 pigments whereas Baltic Sea fishes had pure A1, pure A2 or a mixture of these chromophores. Freshwater fish species had either an A1/A2 mixture or a pure A2 pigment (I & II). All opossum shrimps appeared to have pure A2 pigments as judged by the shape of the absorbance spectra, except *M. diluviana* which appeared to use mixtures of A1 and A2 in different proportions (IV). However, the validity of using vertebrate A1 and A2 spectral templates for judging chromophore content in invertebrate visual pigments has never been firmly established by tests on a wide variety of pigments (see Discussion).

Rod absorbance spectra of the Baltic subspecies or populations of the primarily marine species herring (*Clupea harengus membras*), sand goby (*Pomatoschistus minutus*), and flounder (*Platichthys flesus*) were long-wavelength-shifted compared to their marine populations (I: Table 1). The shifts are consistent with adaptation for improved quantum catch as well as improved signal-to-noise ratio of vision in the Baltic light environment. Since there was no chromophore change (no detectable admixture of A2), this has apparently been achieved by evolutionary tuning of the opsin.

A more detailed analysis of differences in absorbance spectra between and within populations was conducted using the sand goby as a model species (II). Four allopatric populations from the Baltic Sea (B), Swedish west coast (S), English Channel (E), and Adriatic Sea (A) were examined. Rod $\lambda_{\text{max}}$ differed between populations in a manner correlated with differences in the spectral light transmission of the
Table 1: Spectral shifts in the $\lambda_{\text{max}}$ values of rods of flounder, sand goby, herring / Baltic herring, and rhabdoms of *Mysis relicta*. For example, the absorbance spectrum of Baltic herring is red-shifted by approx. 10 nm compared with Scottish herring.

<table>
<thead>
<tr>
<th>Species</th>
<th>Light environment and the spectral shift in $\lambda_{\text{max}}$ value</th>
<th>Freshwater</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marine</td>
<td>Brackish</td>
</tr>
<tr>
<td>Flounder</td>
<td>Norway $\rightarrow$ Baltic + 2 nm</td>
<td></td>
</tr>
<tr>
<td>Sand goby</td>
<td>Adriatic $\rightarrow$ Baltic$_{\text{All}}$ + 5 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adriatic $\rightarrow$ Baltic$_{\text{TypeII}}$ + 7 nm</td>
<td></td>
</tr>
<tr>
<td>Herring / Baltic herring</td>
<td>English $\rightarrow$ Baltic$_{\text{All}}$ + 2 nm</td>
<td></td>
</tr>
<tr>
<td><em>Mysis relicta</em></td>
<td>English $\rightarrow$ Baltic$_{\text{TypeII}}$ + 5 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scotland $\rightarrow$ Baltic + 10 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baltic $\rightarrow$ Lake$_{\text{Pääjärvi}}$ + 25 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baltic $\rightarrow$ Lake$_{\text{Vuohijärvi}}$ + 25 nm</td>
<td></td>
</tr>
</tbody>
</table>

respective water bodies ($\lambda_{\text{max}}$ (A) $\approx$ 503 nm, $\lambda_{\text{max}}$ (E and S) $\approx$ 505-506 nm, $\lambda_{\text{max}}$ (B) $\approx$ 508 nm). A distinguishing feature of Baltic sand gobies was the wide within-population variation of $\lambda_{\text{max}}$ (505.6-511.3 nm).

The second major model taxon chosen for this study, the *M. relicta* species group (IV), was of interest especially for extending the study of adaptation of dim-light visual pigments outside the restricted family of vertebrate pigments. In *Mysis relicta*, *sensu stricto*, the visual pigments of the two lake populations studied were similar ($\lambda_{\text{max}} \approx 555$ nm), but significantly red-shifted compared with the Baltic Sea populations (at $\approx 530$ nm) and with Baltic populations of *M. salemaai* (at $\approx 525$ nm) (Table 1). Since all these pigments had the same chromophore (judged by vertebrate templates to be A2), the lake/sea difference indicates adaptive evolution of the opsin. In the North American *M. diluviana* populations studied, $\lambda_{\text{max}}$ varied in the range 505-529 nm and the shapes of spectra suggested varying A1/A2 chromophore proportions.

In no fish species of freshwater origin, whether living in lakes or in the Baltic Sea, any significant opsin-based spectral shifts were found between populations. Only the A1/A2 chromophore ratio varied between populations, which still allowed significant spectral differences between populations in some species (I).

**Quantum catch and signal-to-noise ratio of dim-light vision as measures of adaptedness (I & II)**

Spectral tuning of pigments to maximise the signal-to-noise (SNR) ratio near the absolute threshold for seeing is an
optimization task, where possible increases in quantum catch (QC) must be weighted against possible increases in noise (see paragraph 2.3.3 above). The relative performance of a rod visual pigment was theoretically calculated as function of $\lambda_{\text{max}}$ for five different light environments ranging from pure ocean water to humus-rich lakes (JI, JII, B, BP, and L$_T$) first, assuming that QC is the relevant measure, second, assuming that SNR is the relevant measure (I). QC was calculated by convolution of the A1 or A2 visual pigment templates (Govardovskii et al 2000) with the respective light environment spectra. The total quantum catch in each case is proportional to the integral of the convolution spectrum across wavelengths, i.e. the area under each curve. In many cases, spectral shifts of even few nanometers turn out to bring functionally significant changes in QC. Quantum catches for sand goby, herring/Baltic herring, flounder, and $M. \text{relicta}$ are given in Table 2.

Table 2: Quantum catch improvements (QC %) achieved through the red-shift of Baltic sand goby, herring and flounder rod pigments compared with the marine pigments in the Baltic Sea light spectrum, and for “Lake” $Mysis \text{relicta}$ compared with Baltic $Mysis \text{relicta}$ in the Lake Pääjärvi light spectrum. In sand goby the QC improvement has been calculated both for the grand mean of all Baltic specimens, on the other hand specifically for the long-wavelength shifted TypeII.

<table>
<thead>
<tr>
<th>Species/ $\lambda_{\text{max}}$ shift (nm)</th>
<th>Light environment/ (quantum catch improvement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand goby</td>
<td>Adriatic Sea → Baltic Sea</td>
</tr>
<tr>
<td>(All +5nm) (TypeII +8nm)</td>
<td>≈ 15% (All)</td>
</tr>
<tr>
<td>Herring/ Baltic herring (+12nm)</td>
<td>≈ 10% (Typell)</td>
</tr>
<tr>
<td>Flounder (+2nm)</td>
<td>≈ 13% (Typell)</td>
</tr>
<tr>
<td>Mysis relicta (+25 nm)</td>
<td>≈ 25%</td>
</tr>
<tr>
<td></td>
<td>≈ 5%</td>
</tr>
<tr>
<td></td>
<td>≈ 115%</td>
</tr>
</tbody>
</table>
Table 3 Optimality intervals of A1 and A2 visual pigments in different light environments. Light spectrum JI (Jerlov 1976; depth 600 m) is applicable to the Adriatic Sea, JIII (Jerlov 1976; depth 90 m) to the coastal Atlantic water, B (Lindström 2000; depth 20 m) to the open Baltic Sea, BP (Lindström 2000; depth 10 m) to the Pojoviken Bay of the Baltic Sea, and LT to the Finnish Lake Tuusulanjärvi (paper I; depth 4 m).

<table>
<thead>
<tr>
<th>Light environment</th>
<th>Optimal interval range (nm)</th>
<th>A1 pigments</th>
<th>A2 pigments</th>
</tr>
</thead>
<tbody>
<tr>
<td>JI</td>
<td>430-470</td>
<td>420-470</td>
<td></td>
</tr>
<tr>
<td>JIII</td>
<td>460-520</td>
<td>450-520</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>500-560</td>
<td>490-560</td>
<td></td>
</tr>
<tr>
<td>BP</td>
<td>520-585</td>
<td>510-585</td>
<td></td>
</tr>
<tr>
<td>LT</td>
<td>595-635</td>
<td>575-635</td>
<td></td>
</tr>
</tbody>
</table>

For the SNR calculation, noise was assumed to increase as function of $\lambda_{\text{max}}$ according to the model of Ala-Laurila et al. (2004a). The “optimal interval” for $\lambda_{\text{max}}$ in each environment was defined as the interval between the QC and the SNR maxima. The short-wavelength limit of the optimal range is set by SNR. Arguably, this is the relevant measure in extreme dim-light conditions, when spontaneous thermal activations of visual pigment molecules may be the dominant limitation to seeing. The long-wavelength limit is set by QC, which tells how well the pigment is able to utilize the available light. Arguably, QC is the relevant measure at slightly higher light levels, where thermal pigment activations become insignificant compared with photon fluctuations. Table 3 gives the theoretical optimal intervals for A1 and A2 pigment in the five “model” light environments (JI, JIII, B, BP, and LT). The width of the optimal interval varies from 40 nm to 75 nm and is larger for A2 than for A1 pigments. Optimal intervals also tend to overlap: visual pigments with absorbance maxima at 500-520 nm are theoretically suitable in both the coastal marine (JIII) and in the Baltic Sea light environments. All the Baltic Sea fishes studied, except one, had rod pigments with $\lambda_{\text{max}}$ falling within the optimal interval. That was true of most other species as well with respect to the optimal interval in their respective light environment.

Opsin gene sequences of closely related goby species and sand goby populations (III)

Rod absorbance spectra were related to the rod opsin sequences of individual sand gobies from four allopatric populations (Baltic, Swedish, English, and Adriatic; III). Substitutions were identified using the English sand goby
sequence of Archer et al. (1992; EMBL/GenBank X62405) as reference and interpreted using two related rod pigments, those of the Adriatic marbled goby Pomatoschistus marmoratus (λ_{max} ≈ 507 nm) and the Baltic common goby P. microps (λ_{max} ≈ 516 nm) as outgroups.

Altogether, nonsynonymous changes in the DNA sequence implying amino acid substitutions between goby species were found in 15 different amino acid positions of the opsin gene (Figure 7). All goby species and even some of the sand goby populations could be distinguished by the specific amino acid sequence. Only four substitution sites were unique to a single goby species or population. Most substitutions were located at sites that varied in several species or populations. Eight of the substitutions were identical in common and marbled goby. Adriatic sand gobies had substitutions, although different, at the very same sites (214 and 217) as had marbled and common gobies.

The opsin sequence of all E individuals was identical to the reference, whereas the S and B fish all had the substitution N151N/T or N151T. The Baltic gobies showed within-population polymorphism correlated with differences in the individual absorbance spectra, the sequence of individuals with λ_{max} at ≈ 506-508 nm (short-wavelength type, Type I) being identical to S, whereas those with λ_{max} at ≈ 509-511 nm (long-wavelength type, Type II) additionally had substitution F261F/Y in the sixth helix close to the chromophore. This substitution (Y261) was also present in the common gobies, which have a long-wave-shifted rhodopsin (λ_{max} at ≈ 516 nm).

Point mutations in the protein-coding regions are of two types: nonsynonymous (those that change the amino acid) and synonymous (those that do not change the amino acid). The number of nonsynonymous and synonymous mutations were calculated for each goby population or species and compared to the total number of 354 amino acids of the goby rhodopsin gene. The rod opsin gene of common goby, marbled goby, Adriatic sand goby, Baltic sand goby Type II, and Baltic sand goby Type I or Swedish sand goby diverged from the reference opsin by 9.3 %, 7.3 %, 3.1 %, 1.4 %, and 0.3 %, respectively.
Fig. 7 Sand goby rod opsin with the amino acid substitutions of all goby species and populations studied here marked by blue and red circles. The 2-D model is modified from that of Hargrave et al (1983) for bovine rhodopsin. The reference English sand goby sequence is from NCBI databank (X62405). The substituted residues are explained in the table below the picture. Stars mark K296, to which the chromophore is bound by a protonated Schiff base, and its negative counterion E113. Abbreviations and the number of individuals are: SGE: English sand goby (N = 3); SGS: Swedish sand goby (N = 1); SGBI and SGBII: Baltic sand gobies Type I and Type II (N = 4 + 4); SGA: Adriatic sand goby (N = 5); MGA: Adriatic marbled goby (N = 2); CGB: Baltic common goby (N = 2). The $\lambda_{\text{max}}$ values (± SEM) are given for the sequenced individuals in the Table below the picture.
6 Discussion

Visual pigment adaptation (I-IV)

It is an old and almost self-evident idea that visual pigments are spectrally “adapted” to the ambient light to provide good conditions for the detection and recognition of stimuli important to the animal’s survival and reproduction. However, much of the large body of evidence is based on comparisons between groups that have diverged at least millions of years ago. The present thesis is based on the idea of bridging the gap between ecological processes acting “here and now” and long-term evolutionary changes. How quickly do the pigments that underlie all vision at the lowest light levels and are therefore necessarily under strong selection pressure change for better performance in a new photic environment? What is good performance? How large spectral shifts can be achieved how quickly, and by what molecular means? While the concept is straightforward, even short evolutionary times (in this study ca.10^4 years) will encompass great uncertainties e.g. about the actual light environments that have been encountered by the populations through history. The ideas discussed in this thesis are summarized in Figure 8.

Fig. 8 Basic determinants of the spectral tuning and signal-to-noise ratio of visual pigments. The two main functional variables, spectral sensitivity and thermal stability, are interdependent: a spectral shift towards longer wavelengths implies lower activation energy of the pigment, and thus a higher rate of thermal activations (noise). The variables may be tuned by changing either of the two main components whose interactions determine the function of the visual pigment: (1) by changing the ratio of chromophores A1 and A2, or (2) by mutation of the opsin gene resulting in changes in the amino acid sequence.
The paired chromophore system (A1/A2) present in many freshwater and brackish sea fish species (Bridges 1972, Bowmaker 1995) provides spectral flexibility on a physiological time scale, changing with season (Dartnall et al 1961), age (Bridges and Yoshikami 1970, Villermet and Weale 1972), location and migration (Beatty 1975) and habitat (Munz and McFarland 1973). For example, an A1 pigment with \( \lambda_{\text{max}} \) at 500 nm has an A2 pair with \( \lambda_{\text{max}} \approx 525 \) nm, and between these limits, \( \lambda_{\text{max}} \) can be continuously increased by increasing the percentage of A2 in chromophore mixtures. The presence or absence of the system (i.e., of the enzyme needed to produce the A2 chromophore) is an evolutionary fact, maybe related to the degree of constancy/variability of the photic environments the animal is likely to encounter during its life time. Truly marine environments tend to be spectrally stable (apart from being blue), and the A2 option is used only by very few marine species. By contrast, freshwater spectra are strongly affected by dissolved organic and inorganic matter, which makes them variable (apart from being generally more long-wavelength-biased). Thus most freshwater fishes have the A1/A2 system, as do diadromous fishes (Bridges 1972, Temple et al 2006, Jokela-Määttä et al 2007, Toyama et al 2008). The fact that we found no indication of opsin-based spectral shifts between lake and Baltic populations among the species studied in paper (I) may suggest that having recourse to chromophore-based tuning lessens the evolutionary pressure for opsin-based tuning. Admittedly, very small opsin-based shifts will be more difficult to detect when chromophore varies. We cannot exclude that the genetic isolation of some of the lake and Baltic populations studied may be incomplete.

In most marine fishes, A2 has either never been present or has been lost secondarily. In spite of its potential value for some situations, maintenance of the required enzyme system may be costly, and the usefulness of A2-based tuning is diminished by its greater noisiness (Donner et al 1990, Ala-Laurila et al 2007). The three species of marine origin studied here (I-III) had only A1 pigment (within our measurement accuracy). In all three, the Baltic populations/subspecies showed long-wavelength shifts of the rod pigment, which must therefore be due to amino acid substitutions in the opsin. These are consistent with evolutionary adaptation to more long-wavelength-biased environments during postglacial history. However, especially in the flounder, the shift was very small (although statistically significant), even smaller than caused by any known single amino acid substitution. It may indicate that Baltic flounders are heterozygous and express opsin mixtures from two alleles.

The basic measure of “spectral match” is quantum catch (QC). Surprisingly small shifts in \( \lambda_{\text{max}} \) may give quite significant increases in QC. For the sand goby (Pomatoschistus minutus) the QC advantage from red-shifting the rod visual pigment by 5 nm in the Baltic Sea (to 508 nm compared with 503 nm in the Adriatic Sea) is nearly 20%. The QC advantage of the Baltic herring (Clupea harengus membras) from using the 512 nm pigment rather than the marine 502
nm pigment is quite substantial, 35%. The Lake Pääjärvi *Mysis relicta* gain 115% in QC from red-shifting $\lambda_{\text{max}}$ by 25 nm, compared with what the Baltic pigment would catch in the lake environment.

**For what should the rod pigment be optimized?**

Quantum catch does not reveal the whole truth, since visual detection depends on SNR rather than QC as such (see paragraph 2.3.3). Near absolute threshold the thermal noise generated by the pigment is a limiting factor that should always be considered, and since the rate of thermal activation tends to increase with increasing $\lambda_{\text{max}}$, spectral sensitivity and noise are interdependent. In paper (I), the $\lambda_{\text{max}}$ values that would maximize QC and SNR in some selected model spectral environments were calculated, and the $\lambda_{\text{max}}$ domain falling between these was termed the optimality interval. As explained above, the SNR optimum falls at lower $\lambda_{\text{max}}$ for A2 than A1 pigments, whereas the QC optimum is the same. It was found that all Baltic species studied except one fell within the optimal interval for the Baltic Sea spectral environment. This may not be surprising: although conceptually strict, the analysis provides rather wide intervals. In A1 pigments the width of the interval was from 40 to 65 nm whereas in A2 pigments it was from 50 to 70 nm. The clearest ocean and very reddish lake water had the narrowest optimality intervals. The limits would be further blurred if QC were calculated more realistically taking into account the axial pigment density (thus based on absorptance rather than absorbance). Yet the analysis clearly demonstrates why the “optimal pigment” may often be displaced to shorter wavelengths compared with that which would give the highest QC, in agreement with distributions found in large samples of rod pigments from coastal and freshwater fishes (Lythgoe 1984). It is of course especially interesting, if a species falls outside the optimality interval. The apparent “maladaptedness” of the rod pigment of greater sandeel in the Baltic might be considered in relation to immigration history and lifestyle.

As discussed in paper (I), the “best” position of $\lambda_{\text{max}}$ depends on the task being optimized. If the main concern is to maximize absolute dark-adapted sensitivity, SNR$_{\text{pigment}}$ is the appropriate measure and $\lambda_{\text{max}}$ would be expected to fall near the lower end of the optimality interval. In case the absolute threshold is less important, the actual signal-to-noise ratio in somewhat stronger light will depend on QC, and $\lambda_{\text{max}}$ would be expected to fall near the upper end of the optimality interval. The advantage of catching many photons (high QC) may be even more pronounced at supra-threshold levels, since the speed of vision increases very steeply with increasing supra-threshold intensity or contrast (Donner 1989). This acceleration is more or less independent of the noise cost, because the sharply rising receptor response to higher contrasts quickly rises out of the noise band. The noise will only degrade the temporal precision of the visual latency.

The range of $\lambda_{\text{max}}$ variation of the Baltic Sea fishes studied was over 50 nm, i.e., quite significant considering that the species live in the same geographical and (photic) area. The A1 pigments varied at
least from $\lambda_{\text{max}} \approx 486$ nm (greater sandeel) to $\lambda_{\text{max}} \approx 516$ nm (common goby). The upper limit is at even at longer wavelengths when taking into account A1/A2 mixtures and A2 pigments: $\lambda_{\text{max}} \approx 539$ nm (perch, white bream). The simple conclusion is that animals can manage with quite different pigments, depending on their lifestyle and environmental preferences. Some of the differences may be due to historical or molecular constraints. Moreover, selective pressures operating on animals are never identical, but produce a trade-off between costs and benefits that ultimately influences the fitness of the whole organism. Natural selection in progress carries “adaptation” to a certain point that may still be far from perfection.

**The rate of visual pigment evolution (I-IV)**

The general conclusion from the comparisons of marine vs. Baltic populations of herring, flounder, and sand goby, and especially the Baltic Sea and Lake populations of *Mysis relicta*, is that adaptive evolutionary changes in dim-light visual pigments can occur very quickly. These examples have a time frame of 10000 years or less. The apparent lack of significant opsin polymorphism in the “ancestral” English and Swedish goby populations and in any of the *Mysis relicta* populations indicates that these fast changes are not explained by selection on previously accumulated polymorphism.

On the other hand, the wide variation of $\lambda_{\text{max}}$ in Baltic sand gobies, correlated with polymorphism in the opsin sequence (more red-sensitive individuals with F261F/Y and less red-sensitive individuals with F261F similar to those of the Swedish West Coast or the English Channel) suggests the presence of contradictory selection pressures in the Baltic.

The compelling nature of the spectral changes is further suggested by the fact that similar absorbance spectra have been multiply realized on different genetic backgrounds (quite different opsin sequences). The Adriatic marbled goby and the English sand goby have the same phenotype (the same $\lambda_{\text{max}}$ value), but many differences at the amino acid level (paper III). The interaction of substitutions may have surprising results due to synergy in the right molecular environment.

Likewise, similar 20-25 nm lake/sea shifts in spectral absorbance were found both in *M. relicta* and *M. salemaai*, but the underlying amino acid substitutions are completely different (Audzijonytė et al in prep.). In fact, the molecular basis for the spectral shift in *M. relicta* is an enigma at present.

**Evolutionary snap shots: molecular tuning of visual pigments (III)**

The $\lambda_{\text{max}}$ of a visual pigment with a given chromophore is determined by a single gene. Useful spectral sensitivity especially in dim light is basically a
question of life and death. Thus natural selection will target the gene in unusually direct fashion. This is one reason why opsin is an especially interesting molecule for the study of mutations and their effects. The crystallography and the opsin sequences of various species have broadened the understanding of the function of the visual pigment molecule and how key amino acids in visual pigments work. So what can be said about the relation between amino acid sequence and \( \lambda_{\text{max}} \)?

Amino acid changes are apparently tolerated only at a few sites. In goby species (III) amino acid substitutions occurred more frequently at the same locations of the molecule although according to the neutral theory of molecular evolution most sequence changes in nucleic acids and proteins are selectively equivalent. Nine of these goby substitution positions were identical to the rhodopsin substitutions in African cichlid species (Sugawara et al. 2002). A total of nine amino acid substitutions introduced into the ancestral pigments (Yokoyama 2008) are reported to be those that mainly shift the absorbance spectrum of dim light pigments. In the goby species, however, substitution is found at only one of these sites with aromatic phenylalanine at site 261 replaced by aromatic tyrosine. However, the amino acid match to potential tuning sites is better if we compare our results with the PAML prediction list of Spady et al. (2005). They list four uncharacterized possible spectral tuning sites for RH1 pigments of which two are identical to this thesis (sites: 41, and 299). Previously, site 299 is shown to be involved in small-scale spectral tuning (Fasick et al. 1998). Therefore, we assume that there must be additional spectral tuning sites for dim light visual pigments of goby species. Nevertheless, the interpretation of the goby opsin sequences and MSP data is quite logical if the site 261 is considered to be the only effective spectral tuning site. Only Adriatic sand gobies do not fit this conclusion, albeit nucleotide positions 214 and 217 can explain the measured spectral mismatch. The role of these substitutions in spectral tuning remains to be resolved.

The idea that each of just a few critical substitutions provides a significant shift in \( \lambda_{\text{max}} \) may readily be connected with the older concept of spectral clustering of visual pigments (Dartnall and Lythgoe 1965). However, the theory of critical substitutions underlying spectral clustering is challenged by several of the main results of the present thesis. First, we find \( \lambda_{\text{max}} \) shifts from smaller to larger without indication of discrete steps. Secondly, the underlying opsin sequences (including unpublished results on Mysids) do not show any clear pattern of common “critical” substitutions causing quantized shifts. The evidence for spectral clustering may have to be revisited with attention to possible artifacts. Apparent clustering may also be enhanced if data are collected mainly from certain light environments, over-representing species around certain \( \lambda_{\text{max}} \) values. Moreover, pronounced shifts (of at least \( \sim 5 \) nm) are those that can be more plausibly measured.

Synonymous and nonsynonymous substitutions between different
populations of the same species were discovered in sand goby. The resulting spectral differences are consistent with spectral differences between the geographical locations of the habitats. The Baltic population has until recently been considered to be similar to the other Atlantic sand goby populations (Gysels et al 2004). One advantage of the combination of accurate MSP with opsin sequencing in identified individuals is that small effects of amino acid differences can be revealed.

The spatially and temporally varying light environment in the Baltic Sea possibly maintains the polymorphism of the opsin gene in sand goby population. The two types of the opsins were present in the same micro-habitats since both types where caught when sampling fishes. Individuals spectrally similar to the English population had “normal” F261 while red-shifted (≈ +5 nm) individuals had the mixture F261F/Y presumably coexpressed in all rods. This substitution (Y261) is also present in common gobies, which have approximately 10 nm red-shifted rod absorbance spectrum compared with that of the English sand goby population. The same substitution is also known to red-shift human cone spectra (Merbs and Nathans 1993) and is present in the rhodopsin of Mexican Blind Cavefish (Yokoyama et al 1995).
7 Conclusions

(1) The degree of adaptedness of dim light visual pigments to the prevailing spectral environment can be assessed by measures of quantum catch (QC) and signal-to-noise ratio (SNRpigment). SNR peaks at lower $\lambda_{\text{max}}$ than QC and the fact that $\lambda_{\text{max}}$ values are often located at shorter wavelengths than would maximize QC suggests that absolute (dark-adapted) sensitivity is limited by pigment noise. At light intensities somewhat above absolute threshold, QC will be crucial, and it is suggested that the wavelength interval between the SNR and QC peaks may be considered an “optimality interval” for $\lambda_{\text{max}}$ in a given light environment. The rod absorbance spectra of the Baltic Sea fishes studied (save one) fell in this interval.

(2) The absorbance spectra of the rod visual pigment differed between marine and Baltic Sea populations of the sand goby, the flounder, and the Baltic herring, indicating evolutionary adaptation of the opsins to the Baltic Sea light environment. The spectral shifts are consistent with adaptation for improved QC as well as improved SNR in the Baltic light environment. No opsin-based $\lambda_{\text{max}}$ differences were found between freshwater (lake) and Baltic populations, only differences due to variation in the A1/A2 ratio in some. It is suggested that the ability to respond to changes in the environmental light by “simply” altering the chromophore ratio is an advantageous solution in variable light environments.

(3) The spectral conditions, the absorbance spectra of the rod visual pigment, and amino acid substitutions of the rod opsin sequence correlate in a comparison between closely related goby species as well as between different sand goby populations. Within the sand goby population of the Baltic Sea, differences in rod absorbance spectra between individuals could be correlated with a specific amino acid polymorphism.

(4) The substitution F261Y contributes the majority of the spectral shift. The same substitution (phenylalanine for tyrosine) red-shifts the rod absorbance spectrum of common goby by approx. 10 nm compared to that of sand and marbled goby. The proposed co-expression of two alleles, F and Y, in the rod photoreceptors of the Baltic sand gobies is a novel tuning mechanism producing a smaller spectral shift (+ 5 nm).

(5) The visual pigments of the opossum shrimp (*Mysis relicta*) "lake" populations are red-shifted by 25 nm compared with all "sea" populations. These populations have been postglacially isolated from the brackish Baltic Sea in several Finnish lakes, some of which have deep brown water colour. Since only A2 chromophore appears to be present in all populations, the differences must reflect evolutionary tuning of the visual protein, the opsin. The molecular mechanism is still unknown.

(6) Adaptive evolutionary changes in the opsin of dim-light visual pigments can occur very quickly. The short postglacial time frame of 10000 years or less applies to the differences found between marine and Baltic populations of sand goby, herring and flounder, as well as the quite dramatic spectral shift between freshwater and Baltic *Mysis relicta*. This underscores that the spectral composition of the underwater light environment sets a very strong selective pressure for proper spectral tuning of dim-light visual pigments.
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