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The visual pigments of diving tetrapods: Genetic and electroretinographic
investigations of pinnipeds, cetaceans, sea turtles, and penguins

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Oceanography

by

David H. Levenson

Committee in charge:

Professor Gerald L. Kooyman, Chair
Professor Paul J. Ponganis
Professor William F. Perrin
Professor Andrew Dizon
Professor Chris Wills
Professor Gerald H. Jacobs

2004

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DEDICATION

This dissertation is dedicated to Professor Jim Enright, who gave me the motivation and support to begin this project, and to my grandmother, Florence Levenson, who gave me the strength to be able to finish it.

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ACKNOWLEDGEMENTS

The text of Chapter One, in part or in full, is a modified version of the material that appears in Proceedings of the Royal Society of London - Series B: Biological Sciences 270: 673-679. I was the primary researcher and author and the co-author listed in this publication directed and supervised the research which forms the basis for this chapter. This chapter was written in collaboration with A. Dizon.

The text of Chapter Two has been submitted for publication. I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter. This chapter was written in collaboration with S.A. Eckert, M.A. Crognale, J.F. Deegan II, and G.H. Jacobs.

The text of Chapter Three will be submitted for publication. I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter. This chapter was written in collaboration with M.A. Croganle, P.J. Ponganis, J.F. Deegan II, A.Dizon, and G.H. Jacobs.

The text of Chapter Four will be submitted for publication. I was the sole researcher and author.

Many people have been involved and contributed in various ways to the completion of this dissertation. First, I would like to thank my committee members, Jerry Kooyman, Paul Ponganis, Bill Perrin, Andy Dizon, Chris Wills, and Jerry Jacobs. All six members have shown unswerving commitment to this project, and to my successful completion of the doctoral program. It is their support that has enabled me to complete this work. Similarly, I would like to thank all my collaborators,

including those listed above, Mike Crognale, Jess Deegan II, and Scott Eckert, as well as Torre Knower, Robert Van Dam, Greg Marshall, Kathi Ponganis, Roshy Tajrishi, and Aimee Lang, who have worked with me on these projects or other research that I have been fortunate enough to be involved with during my years at SIO.

Financial support for this research has come from a variety of sources, evolving over time to meet the specific needs of various projects. I would like to thank Dr. Dave Checkley for his willingness to support me as a SeaGrant trainee during my first year, especially knowing as he did that I would be largely occupied with my coursework and preparation for departmental exams. Further graduate support was provided through my unofficial advisor, Paul Ponganis, who allowed me the wonderful opportunity to work with him on his Antarctic research program, despite my initial non-physiologist status. Funding for Paul's research, including my graduate student stipend was granted by the National Science Foundation, Office of Polar Programs (NSF 9814794) to PJP, though it was the time spent in the field with Paul, Jerry K., and all the others involved with this program, including our fascinating subjects the emperor penguins, that made this such a special research experience. During this time I also received several small grants from Sigma Xi, and The American Museum of Natural History as well as some interim stipend support from the Marine Biology Research Division of SIO that allowed me to begin my dissertation research. The latter half of my research was supported from two sources. First, I was contracted through Richard Brill of the National Marine Fisheries Service, Honolulu, HI to research the visual capabilities of sea turtles (Chapters 2, 4) as part of a program to reduce sea turtle by-catch in Hawaiian long-line fisheries. Second, I was fortunate to have been chosen for a scholarship from the ARCS foundation (Achievement Rewards for College Scientists). The generous award of this

scholarship came as a surprise to me, and even more so as it was maintained throughout the final three years of my research, making much of the work described here possible. Finally, I must thank Andy Dizon and Bill Perrin for most generously allowing me use of laboratory and office space at the Southwest Fisheries Science Center (SWFSC), where the majority of my dissertation research was completed.

In addition to direct assistance, I have received support from a variety of other organizations and individuals. Tissue samples and live animals were provided for these studies from numerous sources. First and foremost, I would like to thank SeaWorld, San Diego for their many different contributions to this project. Live pinnipeds and sea turtles were provided exclusively by SeaWorld, as were many of the tissue samples of pinnipeds, sea turtles, and penguins used in the genetic analyses. The animal husbandry and veterinary staff of SeaWorld were always helpful and thoughtful throughout my many interactions with them over the course of this work. Additional samples were provided by the California Department of Fish and Game, the Alaska Department of Fish and Game, the U.S. Fish and Wildlife Service, the National Marine Fisheries Service (La Jolla, CA, Galveston, TX, and Honolulu, HI), Dr. Siniff's weddell seal research program (McMurdo Sound, Antarctica), and the Center for Reproduction of Endangered Species (CRES).

I am extremely thankful to Luana Galver, who gave me instruction and insight into to the sometimes mysterious procedures of polymerase chain reaction (PCR) and cycle-sequencing. Carrie LeDuc was subsequently essential in setting up the mRNA specific procedures, as well as general troubleshooting in later stages of this research. Rick LeDuc, Milmer Martinez, Nick Kellar, John Hyde, Cindy Taylor, Aimee Lang, and everyone else at the Southwest Fisheries Science laboratory were also helpfully involved, whether it was sitting through discussions of procedural problems, data

analysis, or just everyday non-sense, I was always made to feel welcome at SWFSC, and received nothing but thoughtful support and assistance. At SIO, where the pinniped ERG experiments were completed, Pat Fisher, Torre Knowler, Roshy Tajrishi, Nick Sossin, and especially Christine Levenson were always cheerful and diligent in the often exhausting care and maintenance of live animals at the Scholander Hall facility.

Finally, on a personal note, I would like to thank my family and friends, most of who were not directly involved in this research, but have given me the personal support necessary to complete this work. In particular, I would like to thank my father, Robert Levenson, who has very quietly provided me with nothing less than everything I have ever needed. His support, financially, intellectually, and emotionally forms the cornerstone of this achievement. I would also like to thank my mother, Margot, stepmother, Susan, and brother, Mark for continuous and unflagging interest, support, and encouragement. Thanks also to all my friends, who helped me surf, ski, sail, golf, and travel away my concerns, and thereby maintain my sanity throughout these many years of research. Last, and certainly not least, I would like to thank my beautiful wife, Christine. You have been with me through every day, every moment, of this long journey. Thank you.

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ABSTRACT OF THE DISSERTATION

The visual pigments of diving tetrapods: Genetic and electroretinographic investigations of pinnipeds, cetaceans, sea turtles, and penguins

by

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Doctor of Philosophy in Oceanography

University of California, San Diego, 2004

Professor Gerald L. Kooyman, Chair

To evaluate how terrestrially descended, marine-living tetrapods have adapted their vision for life in the ocean environment, the rod and cone visual pigments of diving turtles, birds, and mammals were examined. Genetics was used primarily. When possible, *in situ* electroretinography (ERG) was also used.

Of the three visual pigments possessed by most mammals, cetacean rod and middle/long-wavelength sensitive (M/L) cone visual pigments had previously been shown to be shifted towards the wavelengths (λ) of light that predominant in marine habits, $\lambda \sim 475$ nm. The genetic sequences of the short-wavelength sensitive (S) cones of numerous cetaceans were examined from nuclear DNA. The distribution of missense mutations found in their S cone opsin genes indicates that all members of the cetacean order lack S cones. Genetic evaluation of the retinal mRNA of seven pinniped species revealed that these mammals also lack S cones. The convergent loss of S cones in pinnipeds and cetaceans suggests that this loss may be adaptive for

vision in the marine environment. Rod and M/L cone pigments of pinnipeds were also examined. In contrast to the cetaceans, one pinniped, the deep-diving northern elephant seal was found to possess a short-wavelength-shifted visual pigment, its rod pigment. The rod and M/L cone visual pigments of the other pinnipeds were similar to those of terrestrial carnivores. An ERG investigation of three pinnipeds including the elephant seal supported the genetic results.

Finally, the rod visual pigments of five sea turtle and three penguin species were examined with genetics. None was found to be markedly marine-adapted. Additional examination of cone sensitivity in sea turtles with ERG indicated limited departure from the terrestrial condition. Clearly, the cetaceans are the most marine adapted of the groups examined. The pinnipeds and sea turtles showed limited adaptation, and the penguins were not apparently different from terrestrial birds. As the cetaceans are the only completely aquatic lineage among these groups, it seems that the terrestrial life-stage may still be of selective significance to some or all of the other groups. Particularly in the case of the sea turtles and penguins, which are highly photopic by ancestry, phylogenetic inertia may also limit the plasticity of the visual systems in adapting for vision in the marine environment.

I. GENERAL SUMMARY

The following is a general summary of the work presented here, including a general discussion of the factors involved in marine vision and a non-specialist summary of the results of the following four chapters of the dissertation. A bibliography is included at the end of the summary that lists most of the major works that were used in the compilation of this overview. However, other than referrals to appropriate chapters in this dissertation where specific discussions may be found, no references will be used within the summary.

The visual environment experienced under water is fundamentally different from that on land. Light is rapidly absorbed and scattered by sea water, so that with increasing depth, ambient illumination is progressively reduced and becomes increasingly monochromatic. Maximum transmission of light in clear seawater occurs in the "blue-green" region of the spectrum (in human color terms) at wavelengths (λ) between 470 and 480 nm. Consequently, to increase visual sensitivity many organisms that inhabit deep marine environments have modified aspects of their visual systems to have peak sensitivity in this region of the spectrum, some 20⁺ nm shorter in wavelength than the peak sensitivity of most terrestrial animals. This trend is particularly well documented in fish, where the degree of the short-wavelength shift can be seen to correlate with the depths inhabited by different species. Shallower mesopelagic inhabitants (depths of 100-400 m) have peak sensitivity from 485-495 nm, while deeper mesopelagic and bathypelagic species (depths of >400 m) may have sensitivity shifted further to wavelengths of 475-480 nm. In cetaceans, a similar increase in short-wavelength shift has been documented in the deeper diving members of that mammalian group.

While this trend in visual sensitivity is quite logical and has been found in a wide range of species, there are still quite a few groups of animals that have not been examined in this context. Of particular interest are those animals that have descended from terrestrial animals but now spend most or all of their time in the marine environment. A variety of animals have re-invaded the aquatic environment in this manner. Sea turtles diverged from other land-based turtles over 150 million years ago (MYA). The penguins diverged from other birds and began their dramatic adaptation for life in the sea around 65 MYA. Several mammalian lineages have also re-invaded the marine environment. Among these are the cetaceans, diverged from terrestrial ungulates ~60 MYA, and the pinnipeds, descended from terrestrial, bear-like carnivores between 25-38 MYA. All these animals exhibit a wide variety of adaptations for life in the marine environment. Many of these adaptations come at the expense of terrestrial abilities; thus, it is interesting to see how factors such as phylogeny, behavior, and life history have affected the convergent evolution of vision in these secondarily marine taxa.

The general organization of the visual systems of vertebrates is relatively consistent. Light enters through the transparent cornea at the front of the eye and then passes through the pupillary opening in the iris where it is then focused by the lens onto the retina at the back of the eye. Within the retina there are several different cell types. The transduction of light into electrochemical signals takes place exclusively in the photoreceptor cell layer at the back of the retina. There are two photoreceptor types, distinguished initially by their shape, rods and cones. The outer segments of these photoreceptors contain light absorbing molecules known as visual pigments that are directly responsible for the transduction of incident photons (light) into electrochemical neural impulses. While additional neuronal interactions that occur

within the retina can have significant effects upon visual functioning, no visual sensation can occur without transduction at the photoreceptor layer; it is the ultimate limit to visual sensitivity. Thus, this is where I have focused my investigation.

Each molecule of visual pigment is composed of two elements, a G-protein opsin, and one of two types of light sensitive chromophore, 11-*cis* retinal, or 11-*cis*, 3,4 di-dehydroretinal. When an appropriate photon is absorbed, the chromophore changes from 11-*cis* to an all-*trans* conformation. This instigates a biochemical cascade of events ultimately leading to the electrochemical polarization of the photoreceptor cell and potential subsequent transmission of the impulse to the brain for perception. Most, though not all vertebrates use retinal chromophores; the chromophore substitution to 3,4 di-dehydroretinal has the general effect of broadening the sensitivity of the pigment, and in most cases, shifting sensitivity towards longer wavelengths. For instance, a retinal-based visual pigment with maximum sensitivity (λ_{\max}) at 502 nm has a λ_{\max} of 518 nm when 3,4 di-dehydroretinal is substituted for the chromophore.

Just as the structure of the chromophore can affect the molecule's sensitivity, changes to the structure of the opsin can also affect the spectral sensitivity of the corresponding visual pigment. Opsins are roughly 350 amino acids in length and contain 7 helical trans-membrane regions joined by interhelical loops. The chromophore is bound to the opsin via an Schiff's base linkage to a lysine residue in the 7th trans-membrane region of the opsin, and it resides within the helical structure of the opsin. The electrostatic nature of this interaction dramatically affects the spectral sensitivity of the visual pigment, by affecting the distribution of charge in the chromophore. Similarly, some amino acid changes can further affect the sensitivity of the pigment. However, to affect spectral sensitivity, these changes must be non-

synonymous substitutions, i.e., the replacement of a non-polar, uncharged residue with a charged and/or polar residue, and they must occur within the opsin-chromophore binding region. Recent advances in understanding of the 3-dimensional structure of opsins have facilitated the location of all potentially spectrally significant amino acids, based on their proximity to this opsin-chromophore binding pocket.

Although all opsins are structurally quite similar, genetic evaluation of the genes underlying the opsin proteins indicate that vertebrates ancestrally possessed five different retinal opsin types. Four of these opsins are cone opsins; in nature they are only found within the cone photoreceptors and their activity underlies photopic (bright light or cone-photoreceptor based) visual functioning. The fifth opsin, the rod opsin or rhodopsin, is a derived form of one of the cone opsin types. It is found exclusively in the rod photoreceptors and is the sole pigment involved in scotopic (dim light or rod photoreceptor based) vision. Reptiles and birds, as well as most other vertebrates have maintained most or all five of these retinal opsin types. In the case of reptiles in general and birds in particular, this has led to the development of excellent photopic visual abilities, including the capacity in some cases for tetrachromatic color vision including sensitivity in the ultraviolet ($\lambda < 400$ nm). Within mammals, at most only two functional cone pigments remain. The other two cone types have been lost, presumably as a consequence of the accumulation of detrimental mutations in the opsin genes of their nocturnal therapsid/mammalian ancestors. As it is the cone visual pigments that underlie most forms of color vision, this loss has resulted in mammals possessing only a dichromatic form of color discriminating ability (Note: some primates, including humans have subsequently evolved alternate forms of trichromatic color vision based on a gene duplication of the one of the two cone types).

It is precisely this sort of disparity that makes the comparative investigation of visual pigments interesting. Reptiles are photopically adapted by nature, including the presence of four cone pigments, as well as light filtering oil droplets that increase color resolution, albeit at the expense of sensitivity. In contrast, mammals have only two cone pigment types, and photoreceptor ratios in the retina that vastly favor the rods, up to 100:1 in some cases. These are animals primarily suited for increased sensitivity. However, both of these groups have terrestrial ancestry and have only subsequently re-adapted to life in the sea. It is the goal of this research project to evaluate as best as possible the visual pigment complements of several of these groups of marine tetrapods in an attempt to compare and contrast how they have respectively adapted their visual abilities for use in the marine environment.

Two techniques have been employed in this regard. The nucleotide structures of the visual pigment opsin genes are relatively well known. Thus, it is possible to learn a great deal about these animals using solely genetic techniques. DNA samples from blood or skin, and messenger RNA (mRNA) samples extracted from retinal tissue obtained from recently deceased animals can be used. Both the DNA and mRNA based analyses involve relatively straightforward application of polymerase chain reaction (PCR) and di-deoxy terminator cycle sequencing. mRNA is preferable in some circumstances as it is a record of the visual pigments (opsins) actually produced within the retina. Moreover, long, uninformative intron sequences present in many opsin genes are spliced out of the mRNA. In contrast, as all vertebrates ancestrally possessed five pigments, all vertebrates have five retinal opsin genes, regardless of whether they are functionally expressed in the living organism. Thus, there is also substantial information available in the nuclear DNA of these animals not

present in the mRNA that can be useful in evaluating the evolution of their visual pigment complements.

A second method employed here was the use of rapid-flicker electroretinography (ERG). In this application, live animals are examined. The data provide a measure of the overall *in situ* sensitivity of the eye, which is a reflection of not only the visual pigments, but also the effects of intra-ocular absorption (e.g., lens, cornea) and reflection (e.g. tapeta). In this type of ERG, a contact-lens electrode is placed upon the surface of the cornea to measure the gross-electropotential changes occurring within in the eye. These changes are a reflection of retinal activity, driven principally by the transduction of the visual stimuli by the photoreceptors. For testing, rapidly flickering light is shined upon the retina, and the response of the eye is observed. Thus, by systematically varying the wavelength and intensity of the lights presented, it is then possible to gain information about the spectral sensitivity of the eye. This technique has previously been applied to a wide range of animals, typically to gain information about their photopic visual abilities.

The first project attempted here was a genetic investigation of the short-wavelength sensitive (S) cones of cetaceans. The other two visual pigments of cetaceans, the rod and middle/long-wavelength sensitive (M/L) cone opsins have previously been shown to be modified for use in the marine environment. The rod opsins are shifted in sensitivity from λ_{max} of 499 nm, such as that seen in the cow or deer, down to λ_{max} of 477-492 nm. In fish, deeper diving species showed greater shifts from the terrestrial condition. The M/L cones of the dolphin are also shifted in sensitivity towards the blue-green region of the spectrum (i.e., $\lambda \sim 475$ nm). For this study, the first two exons of the cetacean S cone opsin gene (about 600 of 1050 nucleotides) were sequenced from a variety of species using DNA from skin. 16

species were examined including representatives of 12 of the 14 extant cetacean families. Mis-sense mutations were found in all obtained sequences, indicating that their S cone opsins genes were in fact pseudogenes, i.e., genes that no-longer code for a functional protein. Consequently, all of these cetacean species must lack S cones. This indicates a photopic insensitivity to short-wavelength light and an inability to make cone-photoreceptor based color discriminations, on account of their possession of only a single cone pigment type. In addition, the distribution of these mis-sense mutations indicates that they arose before the divergence of the two sub-orders of cetaceans, the baleen whales and the toothed whales. As all modern species are members of one of these two sub-orders, all modern cetaceans must therefore lack this cone type.

The second series of experiments was directed at examining the rod and cone visual pigments of pinnipeds (seals, sea lions, and walrus). Initially, three pinniped species were examined with electroretinography (ERG) under photopic (cone-photoreceptor based) testing conditions. Results indicated that the photopic responses of these animals were dramatically different from those of their terrestrial carnivore relatives. Using ERG and genetics, dogs and cats have been shown to have two cone visual pigments, M/L and S cones with λ_{\max} of ~ 553 and 440 nm, respectively and two corresponding peaks in photopic sensitivity. The observed photopic responses of the pinnipeds were monotonic, with peak sensitivity at or just above 500 nm for the California sea lion and harbor seal, and around 491 nm for the elephant seal. In fact, these values were much closer to the predicted scotopic (rod-photoreceptor based) sensitivity of these animals. It is possible that pinniped photopic sensitivity is dramatically different from that of terrestrial carnivores. Alternatively, their responses to the flicker ERG examination arose from the rod photoreceptors, despite testing

under conditions previously shown to elicit strictly cone photoreceptor responses in a wide range of other mammals. In either case, these anomalous results motivated further investigation of pinniped visual pigments.

Consequently, a genetic investigation of the rod and cone visual pigments of pinnipeds was undertaken using retinal mRNA. For this study, sample selection was limited by availability of eyes that could be collected from animals that had recently died or been euthanized in rehabilitation or captive facilities. Animals were collected from the Antarctic and arctic regions, as well as from central/southern California. Polar bear and sea otter samples were obtained for comparative purposes, as well as to determine whether either of these mammals had undergone any marine adaptation. Both rod and M/L cone sequences were readily obtained from the retinal mRNA of all samples. No S cone sequences could be obtained from any of the pinnipeds, leading to the conclusion that, like cetaceans, pinnipeds lack S cones. The polar bear and the sea otter had S cones similar to those of other terrestrial carnivores. Except for the rod pigment of the northern elephant seal, all the pinniped, polar bear, and sea otter sequences were functionally identical to those of terrestrial carnivores. The rod pigment of the elephant seal was short-wavelength shifted roughly 15 nm. In view of these results, it was clear that the ERG data were reflective of scotopic (rod photoreceptor) sensitivity of the pinnipeds tested, despite examination under photopic conditions. The exact cause for this unusual response is unclear and warrants further investigation. The absence of wholesale marine adaptation in these species, such as that seen in the cetaceans, suggests additional selective factors other than the need for increased underwater sensitivity have shaped the evolution of visual sensitivity in pinnipeds.

The presence of multiple additional cone pigment types makes similar investigation of penguin and sea turtle species much more difficult than the study of marine mammals. However, for sea turtles, an examination of the photopic visual sensitivity of several live species was possible through collaboration with Sea World, San Diego. Live loggerhead and green turtles were examined using ERG. Results in this case were clearly reflective of cone photoreceptor responses: both species exhibited sensitivity across most or the entire range of the visible spectrum, 400-700 nm, which would not be possible with a single pigment like that underlying the scotopic visual system. In addition, the obtained sensitivity curves had multiple relative maxima, indicative of the presence of multiple photoreceptor types. In general, results indicated that the sensitivity of sea turtles was not as limited as that of the cetaceans or pinnipeds. There was a definite indication of the presence of S cones, as well as probably at least two other cone types. Although color discrimination was not tested explicitly, the presence of multiple cone pigment types suggests that unlike marine mammals, sea turtles probably possess some form of cone-based color vision. However, there were some differences from terrestrial/freshwater turtles suggestive of marine adaptation in sea turtles. In comparison to freshwater turtles, these sea turtles exhibited a reduced range of sensitivity with a general tendency toward short-wavelength shifted sensitivity. Much of this is likely due to the substitution of chromophore in sea turtles to a retinal chromophore from the 3,4 di-dehydroretinal chromophore of freshwater/terrestrial turtles.

The final investigation was directed at the rod visual pigments of sea turtles and penguins, the deepest diving members of reptilian and avian groups. Since light levels are typically very low at depth, it is the rod pigments that would be utilized while diving there. It has previously been determined that two shallow divers, the

green sea turtle and the Humboldt penguin, have rod pigments with λ_{\max} of 502 and 504 nm, respectively. These values are typical of terrestrial animals (though not terrestrial/freshwater turtles, on account of the previously discussed chromophore switch). However, the question remained whether the deeper-diving members of these groups had modified rod pigments. A total of five sea turtle species and three penguin species were examined including the deepest-diving members of both groups. Retinal mRNA was used to obtain the sequences of their rod opsins. Comparative evaluation indicated that none of these animals, not even the deepest divers, had rod opsins that were functionally distinct from those of the shallower-diving species with known sensitivity. Thus, none of these animals have rod pigments adapted for sensitivity in the deep-sea marine environment.

Although at least some adaptations were seen for most species examined here, there were undoubtedly fewer changes than would be expected based on previous studies of fish, cetaceans, and other marine organisms. Several considerations might help explain this discrepancy, and it is worth mentioning each briefly in the context of these results. First, except for the cetaceans, all the species examined still spend some portion of their lives on land. The time may be very limited, as in the sea turtles, which come ashore for only a few hours a year to nest. In other species it is more substantial: pinnipeds and penguins spend weeks or months out of the water to reproduce, nurture their young, and moult. The potential utility of vision during these aspects of their life history may have considerable influence over the evolution of their visual systems.

In addition, some trends in our findings suggest that phylogenetic inertia may also be very important. Both the pinnipeds and cetaceans have lost their S cones, while the sea turtles have not. However, the sea turtles have a much more complex

and well-developed photopic visual system. Their eyes are proportionately small (in comparison to pinnipeds), with consequently limited light gathering capabilities.

Unlike the rod dominated retinæ of pinnipeds and cetaceans, sea turtle retinæ have multiple types of cone photoreceptor that predominate in ratios of up to 5:1 over the rods. In this case, the ancestral design of the sea turtle (and perhaps avian) eye may limit the adaptive utility of specialization of the visual pigments for scotopic function as the eye is already photopically specialized.

Finally, it is important to consider the relatively simplistic approach used in determining what constitutes marine adaptation. Using cetaceans and deep-sea fish as a bench mark by which to compare other species is certainly reasonable, but clearly it has some limitations. For instance, despite being completely aquatic since some time in the Eocene (~35-50 MYA), the third group of marine mammals, the sirenians (manatee and dugong), still have S cone pigments and rod pigments with λ_{\max} of 502 nm. Among fish, there may also be a greater variety of visual pigment complements, even amongst species from similar habitats. Variability may be related to foraging behavior, as with the herbivorous sirenians, intra-species interactions, developmental migration, or even polarization sensitivity, as in the case of the UV sensitivity of some fish. Similarly, for many of the shallow-diving species examined here, particularly those inhabiting coastal habitats, short-wavelength shifted pigments might not be adaptive at all, as some coastal waters have λ_{\max} at 500 nm or above. Clearly there are factors involved in marine adaptation other than maximizing sensitivity to the wavelengths of light that predominate in relatively deep ocean waters that should be taken into account when considering the visual pigment complement of a species in terms of its potential adaptive significance.

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II. CHAPTER ONE

Genetic evidence for the ancestral loss of S cone pigments in mysticete and odontocete cetaceans

Abstract

All mammals ancestrally possessed two types of cone pigments, an arrangement which persists in nearly all contemporary species. However, the absence of one of these cone types, the short-wavelength sensitive (S) cone, has recently been established in several delphinoid cetacean species, suggesting that the loss of this pigment type may be widespread among cetaceans. To evaluate the functional condition of S cones in cetaceans, partial S cone opsin gene sequences were obtained from nuclear DNA for sixteen species representing twelve of the fourteen extant mysticete (baleen) and odontocete (toothed) families. For all these species one or more mutations were identified that indicate that their S cone opsin genes are pseudogenes and thus do not code for functional visual pigment proteins. Parsimonious interpretation of the distribution of some of these mis-sense mutations indicates that the conversion of cetacean S cone opsin genes to pseudogenes probably occurred before the divergences of the mysticete and odontocete suborders. Thus, in the absence of dramatic homoplasy, all modern cetaceans lack functional S cone visual pigments and, by extension, the visual capacities that such pigments typically support.

Introduction

The transduction of light in vertebrate photoreceptors is performed by apoproteins known collectively as visual pigments (Wald 1968). Each visual pigment molecule consists of a G protein-coupled receptor, opsin, and a photosensitive chromophore, 11-*cis* retinal. Molecular genetic analyses of vertebrate opsins reveal that these proteins may be sub-divided into five distinct retinal classes as well as one or more extra-retinal classes (see Yokoyama 2000 for a review). The retinal classes include the RH1 opsins, found in the rod photoreceptors, and four cone-opsin types, S1, S2, M, and M/L. In general, these classes are characterized by the region of the spectrum to which their constituents are most sensitive. For instance, the S1 and S2 classes consist primarily of short-wavelength sensitive (S) cone opsins, while the M/L class contains opsins maximally sensitive in the middle to long-wavelength region of the spectrum (Yokoyama and Yokoyama 1996; Bowmaker et al. 1997; Yokoyama 2000). Phylogenetic analyses of various opsin genes indicate that modern birds, fish, and other non-mammalian vertebrates may possess functional forms of all five retinal opsin types (Bowmaker et al. 1997; Yokoyama 2000). Within mammals, however, a maximum of three opsin genes are present in functional form (Yokoyama and Yokoyama 1996). These are the rod opsins, RH1, and two cone opsin types, S1 and M/L. The other two cone pigment types, S2 and M, were lost some time before the divergence of modern mammals. Behavioral and physiological examinations of a range of species confirm that almost all mammals possess functional forms of these two cone opsin types as well as the corresponding ability for at least dichromatic color vision (Jacobs 1993).

A limited number of mammalian species have been identified as cone monochromats. They lack functional S1 (S hereafter) cones and, therefore, have only

a single functional cone visual pigment type, an M/L cone. In terrestrial mammals, this condition is unusual, although the nature of its distribution suggests it has arisen independently in several different lineages including some primate (Jacobs et al. 1996), rodent (e.g. Szel et al. 1996; Peichl and Moutairou 1998), and carnivore species (Jacobs and Deegan 1992; Peichl and Moutairou 1998; Peichl and Pohl 2000). As essentially all these terrestrial cone monochromats are primarily active in dim nocturnal and/or subterranean habitat, the loss of S cone sensitivity has been attributed to a lack of selective pressure for the maintenance of good photopic (cone-based) vision (Jacobs 1993; Peichl and Moutairou 1998; Peichl and Pohl 2000). In contrast, the absence of functional S cones has recently been documented in seven cetacean species, many or all of which are active in photopic conditions (Fasick et al. 1998; Peichl et al. 2001). Several amphibious mammals have also been found to lack S cones (Peichl and Moutairou 1998; Peichl et al. 2001). Thus, it has been suggested that at least in marine mammals the loss of S cones is adaptive, presumably for vision under water in photopic conditions, and consequently that all cetaceans lack S cone visual pigments (Peichl et al. 2001).

Presently, however, the cetacean species that have been evaluated are limited to closely related delphinoid (dolphin and porpoise) species; no mysticete (baleen) whales have been examined, nor have any of the riverine dolphins or highly pelagic, deep-diving odontocete (toothed) whales. To assess the extent to which S cones have been lost throughout the cetacean order, we have obtained DNA sequences to evaluate the S cone visual pigment genes of a taxonomically diverse selection of mysticete and odontocete cetaceans. We chose a genetic approach as it reduces the potential for obtaining false negative results by positively identifying the mutation(s) underlying the absence of the pigment type (Jacobs et al. 1996; Fasick et al. 1998). Moreover, as

genetic techniques are not limited to examination of live animals or even fresh tissue samples, it is possible to study a much broader range of species, allowing us to examine representatives of nearly every extant cetacean family.

Methods

The sixteen cetacean species evaluated are listed in Table 1; these species represent 12 of the 14 currently recognized cetacean families (Rice 1998). For genetic analysis, three individuals were examined from each species, except *Inia geoffrensis* and *Platanista gangetica*, where tissue availability was limited to a single individual. All procedures were performed on nuclear DNA extracted from frozen skin with lithium-chloride and chloroform (Hillis et al. 1996). Three river hippopotamus (*Hippopotamus amphibius*), the closest living relative of cetaceans (Nikaido et al. 1999), were similarly examined for comparative purposes. Bottlenose dolphin (*Tursiops truncatus*) and cow (*Bos taurus*) were examined solely to obtain comparative sequence data for the region of the first intron. S cone coding sequence data for these species were obtained from Genbank, accession numbers U92557 and U49760, respectively (Chiu et al. 1994; Fasick and Robinson 1998).

Nucleotide sequence data was obtained using polymerase chain reaction (PCR) and the dideoxy-chain-terminator sequencing method (Sanger et al. 1977). For PCR, 1 µl of nuclear DNA (~100 ng/µl) was amplified in a 50 µl mixture containing 37.75 µl of water, 5.0 µl of MgCl₂ polymerase buffer (2.0 mM), 3.0 µl of mixed DNTP's (10 mM), 1.5 µl of sense and anti-sense oligonucleotide primer (10 µM), and .25 units of *Taq* DNA polymerase. Amplification was achieved by placing the reaction through thirty-five cycles of denaturing at 90 °C for 45 s, annealing at 48 °C for 60 s, and extension at 72 °C for 90 s, as well as a final extension period of 5 min at 72 °C. For some reactions annealing temperatures were adjusted between 42 and 62 °C to

Table 1. Nucleotide identity and non-synonymous substitution proportions for partial (~391 bp) hippopotamus and cetacean S cone opsin gene coding sequences in comparison to the homologous bovine coding sequence (accession number U49760).

common name	<i>Genus species</i>	nucleotide identity (coding only)	proportion non-synonymous substitutions		indels	apparently functional?	accession number
			# A.A. subs/# nuc. subs				
river hippopotamus	<i>Hippopotamus amphibius</i>	0.936	* 0.16	(4/25)	0	yes	AF545497
common minke whale	<i>Balaenoptera acutorostrata</i>	0.938	0.50	(12/24)	1	no	AF545490
blue whale	<i>Balaenoptera musculus</i>	0.938	0.46	(11/24)	1	no	AF545484
humpback whale	<i>Megaptera novaeangliae</i>	0.938	0.42	(10/24)	1	no	AF545494
bowhead whale	<i>Balaena mysticetus</i>	0.933	0.54	(14/26)	1	no	AF545491
North Atlantic right whale	<i>Eubalaena glacialis</i>	0.930	0.56	(15/27)	1	no	AF545492
gray whale	<i>Eschrichtius robustus</i>	0.928	0.50	(14/28)	1	no	AF545493
sperm whale	<i>Physeter macrocephalus</i>	0.918	0.44	(14/32)	0	no	AF545482
pygmy sperm whale	<i>Kogia breviceps</i>	0.912	0.50	(17/34)	1	no	AF545486
Indian river dolphin	<i>Platanista gangetica</i>	0.920	0.58	(18/31)	1	no	AF545487
Sowerby's beaked whale	<i>Mesoplodon bidens</i>	0.929	0.48	(13/27)	2	no	AF545496
Amazon river dolphin	<i>Inia geoffrensis</i>	0.921	0.47	(14/30)	2	no	AF545495
fransiscana	<i>Pontoporia blainvillei</i>	0.923	0.53	(16/30)	1	no	AF545483
# common bottlenose dolphin	<i>Tursiops truncatus</i>	0.909	0.57	(20/35)	3	no	U92557
beluga whale	<i>Delphinapterus leucas</i>	0.930	0.44	(12/27)	1	no	AF545485
vaquita	<i>Phocoena sinus</i>	0.936	0.44	(11/25)	1	no	AF545489
# harbor porpoise	<i>Phocoena phocoena</i>	0.928	0.46	(13/28)	1	no	AF545488

previously identified as lacking S cones

*significantly different from cetaceans, $p < .001$

optimize primer performance. PCR products were analyzed by size with ethidium bromide- stained agarose-gel electrophoresis. Desired PCR products were purified and concentrated using Qiagen (Valencia, CA) Qiaquick[∞] PCR purification columns and then sequenced directly using Applied Biosystem's (ABI; Foster City, CA) Big DyeTM chemistry on an ABI 377 auto-sequencer. Both sense and anti-sense primer products were sequenced.

Oligonucleotide primers for PCR and sequencing were 14-22 base pairs (bp) in length, and were selected from conserved regions of the S cone opsin genes of the bottlenose dolphin (Fasick and Robinson 1998), cow (Chiu et al. 1994), and human (accession number AH003620; Nathans et al. 1986). The primers were designed in

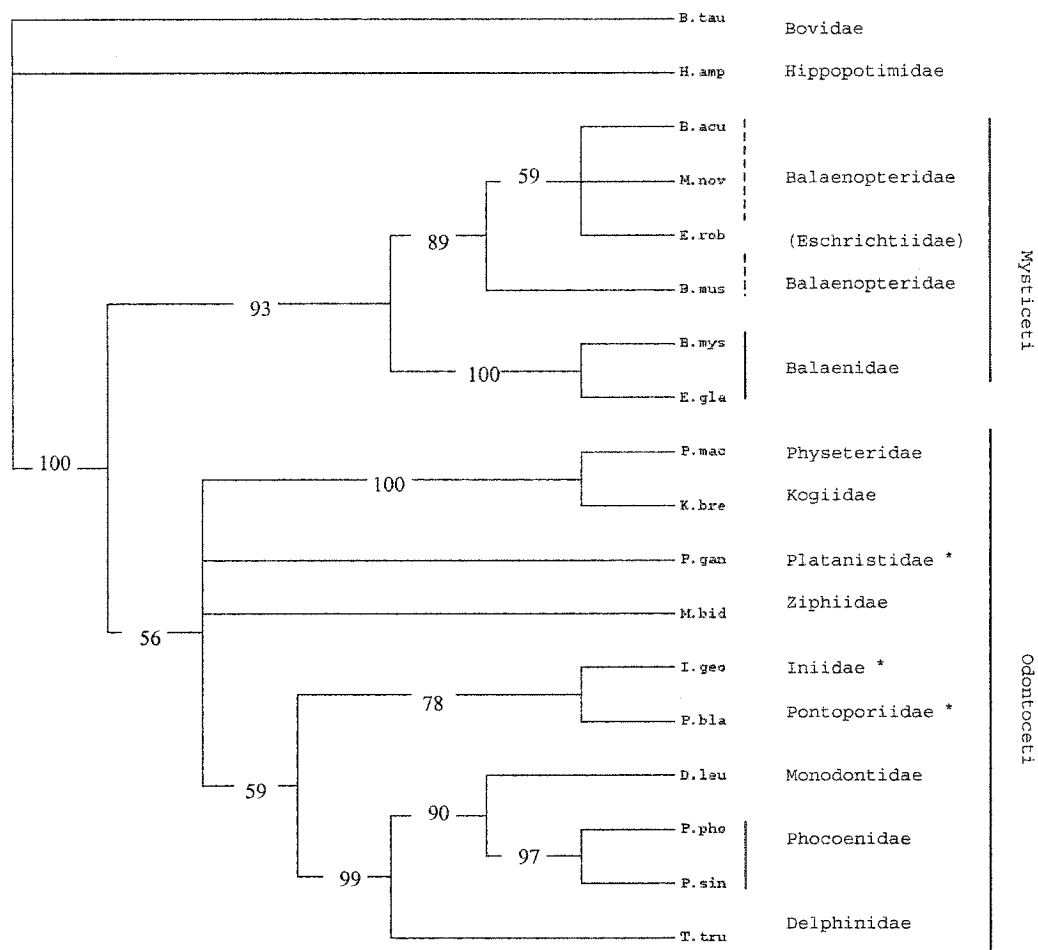


Figure 1. Bootstrap consensus tree of the cetacean, hippopotamus, and bovine S cone opsin sequences obtained using parsimony (1000 replicates). Bootstrap values are shown for each node. Nodes with bootstrap values of less than 50% were collapsed. Current sub-order and familial designations are indicated along the right margin (Rice 1998). * = "river dolphin."

pairs to amplify two overlapping sequence fragments beginning just upstream of the translation initiation codon and including the entire first exon and intron as well as most of the second exon. After the sequences were obtained, introns were identified

by homology using dolphin, bovine, and human S cone opsin sequences. Sequences were aligned for comparison using ClustalW (Thompson et al. 1994) and subsequently checked by eye. Quantitative evaluation of nucleotide and amino acid substitution rates were performed by eye while the sequences were aligned. Phylogenetic analyses, nucleotide identity values, and Jukes-Cantor distances were calculated from the aligned sequences using PAUP (Swofford 2001).

Results

Partial S cone opsin gene sequences of 660-690 bp were obtained for the river hippopotamus and fifteen cetacean species. Sequence data was also obtained for the first intron region of the S cone opsin gene of a sixteenth cetacean species, *Tursiops truncatus* (accession number AF545498), as well as the cow (AF545499). Although each gene was sequenced multiple times for each species, no evidence was found for the presence of markedly different paralagous copies of the putative S cone opsin gene in any individual of any species. Based on an evaluation of apparent polymorphisms in the raw sequence chromatogram data, intra-species variability was less than 1% in all cases. A small number of single nucleotide polymorphisms were observed, but their paucity suggests that they were likely a consequence of allelic heterozygosity rather than the result of whole or partial gene duplication. Regardless of whether single or multiple copies of the S cone opsin gene were present, no polymorphisms were seen for any individual at any of the functionally significant sites described below. A phylogenetic analysis of the complete data set performed using parsimony is shown in Figure 1.

The spliced cetacean and hippopotamus coding sequences exhibited nucleotide identity values with the bovine S cone opsin gene of greater than 0.90 (Table 1). Jukes-Cantor distances reflected similar differentiation: exonic distances between the

cetaceans and cow ranged from 0.063 to 0.103; hippopotamus distance from the cow was intermediate at 0.066. Intronic Jukes-Cantor distances were higher, as expected, and ranged from 0.171 to 0.206, again with the hippopotamus intermediate at 0.179. However, while summary cetacean and hippopotamus mutation rates were apparently similar, there was a distinct difference in the nature of their respective mutational changes. As shown in Table 1, all sixteen cetacean species exhibited significantly higher proportions of non-synonymous substitutions when compared to the hippopotamus (Table 1; Fisher's PLSD, $df=15$, all p values <0.001).

The primary structure of the sequenced cetacean and hippopotamus S cone opsins are shown in comparison to the functional cow opsin in Figure 2. Both the cetacean and hippopotamus sequences contain many of the highly conserved S cone residues (underlined in Figure 2) identified by Chiu et al. (1994). Mis-sense mutations are apparent in all the cetacean S cone opsin genes; none can be seen in those of the hippopotamus or cow. In all six mysticete species, as well as in *Mesoplodon bidens* and *Platanista gangetica*, a mutation in the first exon creates a shift in the reading frame and introduces a premature stop codon after residue 120 (using the bovine S sequence numbering). Furthermore, the substitution E111G, i.e. the substitution of glutamate at residue 111 with glycine, is apparent in all the odontocete species examined, excepting those that possess upstream frame shifting mutations. Residue E111 of the S cone opsin corresponds to residue E113 of bovine rhodopsin, the essential Schiff's base linkage counter-ion that stabilizes the opsin-chromophore bond (Sakmar et al. 1989; Nathans 1990). The detrimental effect of the previously described frame shifting deletion mutations of the bottlenose dolphin, *Tursiops truncatus*, can also be seen (Fasick et al. 1998).

	10	20	30	40	50	60	70	80	90	100	110	120	130	
B.tau	MSKMSEREERF	LLFKNISLVG	PWDGQYHLA	PYWAFLQAV	FMGFVFFVGT	PLNATVLVAT	LRYRKLQPL	NYILVNVSLG	GHVYCFPSVF	IVFITSCYGY	FVFGRHVICAL	EAF	AGCTAGL	VTGWSLAFLA
H.amp	
B.acu	T.L.....	A.T.....	M.....	S.-SL	SSSSPAVMMT	LSSAAMFVLM	RPSNAVQQVM	*	
B.mus	T.....	A.T.....	VS.M	-SL	SSSSPAVMMT	LSSAAMFVLM	RPSNAVQQVM	*	
M.nov	T.....	A.T.....	M	-SL	SSSSPAVMMT	LSSAAMFVLM	RPSNAVQQVM	*	
E.rob	T.....	C.A.T.....	L.M	P	-SL	SSSSPAVMMT	LSSAAMFVLM	RPSNAVQQVM	
B.mys	T.....	V.A.T.....	S.R.....	Q.....	-SL	SSSSPAVMMT	LSSAAMFVLM	RPSNAVQQVM	
E.gla	T.....	V.A.T.....	S.R.....	Q.....	-SL	SSSSPAVMMT	LSSAAMFVLM	RPSNAVQQVM	
P.mac	T.....	S.A.T.....	A.V.....	C.....	V.....	H.....	C.....	G.....	
K.bre	L.YE.....	S.A.T.....	R.H.....	V.....	H.....	..SC.....	G.....	M.....	
P.gan	F.C.D.....	F.A.T.....	M.....	-SL	SSSSPAVMMT	LSSAAMFVLM	RPSNAVQQVM	*	
M.bid	H.....	FN.-SL	SSSSPAVMMT	LSSAAMFVLM	RPSNAVQQVM	*	
I.geo	I.....	S.....	A.T.....	V.....	V.....	H.....	L.....	G.....	
P.bla	T.....	D.F.....	SA.....	A.T.....	S.....	V.....	H.....	CLWPPCL.SG	GLFELYRSRG	DRLVTLGLG	
T.tru	R.....	FCRRTS-EM	RGM.LSIASP	LSGLPPPSL	HGLCLPCLEG	TECHSAGSH	ALOKVAAATQ	LHSGQRIPG	LHLLHLLCLC	RLHHLSWIL	CLRPPCL.SG	GLFEPYRSRA
D.leu	F.....	R.....	R.....	A.T.....	V.....	VI.....	H.....	G.....	
P.pho	F.....	L.....	R.....	A.T.....	V.....	V.....	H.....	G.....	
P.sin	F.....	R.....	A.T.....	V.....	V.....	H.....	G.....	

Figure 2. A comparison of transcribed bovine, hippopotamus and cetacean S cone opsins. Note that non-frameshifting gaps have been inserted as necessary to facilitate comparison. The disruptive effects of frame shifting indel mutations can clearly be seen in many of the cetacean sequences. In all odontocetes not possessing upstream frame shifting mutations, the non-synonymous change E11G (the replacement of glutamate at residue 111 with glycine) which disrupts the chromophore-binding Schiff's base linkage, is also apparent. The boxed area contains the region for which nucleotide data is presented in Figure 3. Residue numbering is based on the bovine sequence. Residues identical to those of the bovine sequence are denoted “.”; residue gaps are denoted “-”; stop codons are denoted “*.”.

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          260          270          280          290          300          310          320          330          340
B.tau  ACTGCATCTT CTCTGCTTC ATCGTCTTCA TCACCAGTTG TTATGGGTAC TTCGTCTCG GCCGCCATGT CTGTGCCCTG GAGGCCCTCC
H.amp  .....T G.....C.. CC...A... T..... A..... T...C..T...
B.acu  ..C.....T G.....C.. .C...AA... T..... A..... T...TT...
B.mus  .....T G.....C.. .C...AA... T..... T.....T...
M.nov  .....T G.T.....C.. .C...AA... T..... A..... T...T...
E.rob  .....T G.....C.. .C...AC... T..... A..... T...T...
B.mys  .....T G.....C.. GC...AA... T..... T.....T...
E.gla  .....T G.....C.. GC...AA... T..... T.....T...
P.mac  .....T G.....C.. .C...A... T..... T...C...T...G...
K.bre  .....T G.....C.. .C...A... T...A...T...C...T...G...
P.gan  .....T G.....C.. .C...A... T...A...T...G...
M.bid  ..T.A...T G.....C.. .C...A... T...A...T...G...
I.geo  .....T G.....C.. .C...A... T..... T...T...G...
P.hla  .....T G.....C.. .C...A... T..... T...T...G...
Y.tru  .....T G.....C.. .C...A... T..... T...T...G...
D.leu  .....T G.A.....C.. .C...A... T..... T...T...G...
P.pho  .....T G.....C.. .C...A... T..... T...T...G...
P.sin  .....T G.....C.. .C...A... T..... T...T...G...

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Figure 3. Nucleotide sequence of a portion of the first and second exons of the S cone opsin genes of sixteen cetacean species, hippopotamus, and cow. Both frame shifting and non-frame shifting nucleotide indels have been used to facilitate comparison. The frequency of nucleotide substitutions in the hippopotamus is similar to that of the cetaceans even though the relative number of amino acid changes is dramatically reduced (compare to Figure 2). Two critical mis-sense mutations are underlined, the four-bp deletion at nucleotides 262-265, and the substitution A to G at nucleotide 332. Nucleotide numbering is based on the bovine sequence. Cetacean and hippopotamus nucleotides identical to those of the cow are marked “.”; nucleotide gaps are marked “-”.

Nucleotide sequence data for the region of the S cone opsin gene containing the majority of these mis-sense mutations is presented in Figure 3. As shown, the point mutation A to G at nucleotide 332 (using the bovine opsin numbering) responsible for the residue substitution E111G is present in all odontocetes, including those with upstream frame shifting mutations that do not exhibit the aforementioned amino acid change. Similarly, the four-bp indel mutation that disrupts the S cone opsin genes of all the mysticetes and two of the odontocetes can be seen at nucleotides 262-265.

Discussion

The results of the phylogenetic analysis of the obtained sequences are in general agreement with currently accepted cetacean systematics (Rice 1998; Nikaido et al. 1999; 2001). The tree structure is also largely consistent with both morphologic and genetic cetacean phylogenies (e.g. Barnes et al. 1985; Nikaido et al. 2001). Our results confirm the recently described paraphyletic status of the river dolphins (Hamilton et al. 2001; Nikaido et al. 2001; Yang et al. 2002) as well as the finding that the superfamily Physeteroidea (families Physeteridae and Kogiidae) and families Platanistidae and Ziphiidae are the three basal branches of the odontocete lineage (Nikaido et al. 2001). The only anomalous aspect of our obtained phylogeny is the placement of the gray whale, *Eschrichtius robustus*, within the balaenopterid family grouping (Figure 1). However, while this result could simply be a consequence of limited sample size, the grouping was moderately robust. Forced grouping of Eschrichtiidae and Balaenopteridae into their respective families raised the number of required steps for the most parsimonious tree from 234 to 244. Indeed, similar findings have been reported elsewhere (e.g. Yang et al. 2002), suggesting further investigation of the relationships of these mysticetes may be merited.

The high degree of sequence identity with the bovine S cone opsin and the presence of many conserved S cone opsin residues confirm that the hippopotamus and cetacean genes sequenced were members of the S(1) cone visual pigment class. In those sequences frame shifted by indel mutations, codons for many of the conserved residues can still be seen within the nucleotide data even though they are no longer reflected in the amino acid translation. The primary structure of the hippopotamus S cone opsin is very similar to that of the functional bovine opsin and contains no apparent mis-sense mutations. Thus it seems likely that, like its close relative the

pygmy hippopotamus (*Choeropsis liberiensis*; Peichl et al. 2001), the river hippopotamus is capable of expressing functional S cone visual pigment. In contrast, all of the cetacean S cone opsin genes appear to be pseudogenes. Every species examined was found to possess one or more mis-sense mutations, and there was no indication from the results of PCR or sequencing analysis that any individual of any of the species examined possessed paralogous functional copies of this gene. The four-bp deletion possessed by all six mysticetes, *Mesoplodon bidens*, and *Platanista gangetica* introduces a premature stop codon that dramatically truncates the encoded S opsin protein, including the elimination of several essential functional regions (Hargrave 1982). In those odontocetes whose genes are not disrupted by upstream frame shifting mis-sense mutations, a single nucleotide substitution in the second exon results in the loss of the glutamate counter-ion. Mutagenesis studies have unequivocally demonstrated that the elimination of this residue results in failed opsin-chromophore bonding (Nathans 1990). In *Tursiops truncatus* and *Pontoporia blainvillei*, additional frame shifting indel mutations are present that further disrupt their respective S cone opsin genes. Consequently, none of these cetaceans are capable of producing functional S cone visual pigment.

The distribution of these mis-sense mutations among the species examined suggests that the loss of the S cone pigment type occurred relatively early in the evolution of modern cetaceans. For instance, the point mutation responsible for the elimination of the glutamate counter-ion is present in all the odontocete species examined, including even the most basal lineage, the physeteroids. For this to occur, the mis-sense character must have been present in the S cone opsin genes of the ancestors of modern odontocetes when the sub-order first began to diverge in the Oligocene, 25-38 million years ago (MYA; Barnes et al. 1985; Nikaido et al. 2001).

Similarly, nucleotides 262-265 have been deleted from the S cone opsin genes of every individual of all six of the mysticete species that were examined. This suggests that this character was fixed in the S cone opsin genes of the ancestors of modern mysticetes when they first diverged in the late Oligocene or early Miocene 15-25 MYA (Barnes et al. 1985; Nikaido et al. 2001). Since the distribution of these two mis-sense mutations indicate that the loss of S cones is ancestral to both odontocete and mysticete cetaceans, then in the absence of dramatic homoplasy all modern cetaceans must lack S cones. The high proportions of non-synonymous substitutions seen in the species examined, a phenomenon characteristic of pseudogene evolution (Li et al. 1985), support this conclusion that these genes have been evolving outside of functional selection for some time. By comparison, the hippopotamus, which has accumulated a similar number of nucleotide substitutions as these cetaceans, has less than half the number of non-synonymous changes and exhibits no apparent mis-sense mutations.

Indeed, it is not possible to determine how far back the original loss of S cones occurred in cetaceans. The apparently identical four-bp deletion present in all six mysticetes and two odontocetes (Figure 3) raises the possibility that the loss of S cones may have even preceded the separation of the two cetacean sub-orders. Yet, there was no single mis-sense character found in all the cetaceans examined that would unequivocally support this claim. Similarly, it is also not possible to determine from these data whether any of the mis-sense mutation(s) discussed here were responsible for the initial conversion of cetacean S opsin genes to pseudogenes. These mis-sense mutations might easily have arisen after the gene had already converted to a pseudogene and was released from the constraints of functional selection. All that the presence and distribution of these mutations demonstrates is that the S cone opsin

genes of modern cetaceans no longer encode viable visual pigment proteins. These results are supported directly by reports of the absence of S cones in *Tursiops truncatus* (Fasick et al. 1998) and *Phocoena phocoena* (Peichl et al. 2001), and indirectly by similar findings in five other cetacean species (Peichl et al. 2001).

Cetaceans are the first order of mammals found entirely lacking one of the three ancestral photopigment types. They also represent the only entire order of mammals known to lack the potential for cone-based color vision (Jacobs 1993). While their nearly unique status as exclusively aquatic mammals seems likely to be involved, the adaptive significance of this loss of S cone sensitivity is not immediately apparent. In view of the relatively ancient nature of their loss of S cones, the monochromatic condition of cetaceans might simply be a non-adaptive artifact of their evolution, brought about by random mutation or limited selective pressure to maintain a short-wavelength sensitive cone pigment in the dim conditions found under water. Alternatively, the concurrent loss of S cones in other marine mammals (Peichl and Moutairou 1998; Peichl et al. 2001) suggests that the absence of this cone type is adaptive and somehow beneficial for underwater vision in carnivorous marine mammals. Unlike its terrestrial relatives, the M/L cones of one cetacean, the bottlenose dolphin, are shifted in sensitivity towards the wavelengths of light that predominate underwater (Fasick and Robinson 1998), evidence that cone-based vision continues to be of importance to at least some cetaceans. Moreover, only a single cetacean species, the Indian river dolphin, *Platanista gangetica*, exhibits any apparent reduction in visual anatomy that might be attributed to a lack of need for vision (Herald et al. 1969), and research continues to document the importance of vision for even the deepest diving cetacean species (e.g., Fristrup and Harbison 2002). While several potential benefits for the loss of S cones are conceivable (see Peichl et

al. 2001 for a discussion), we favor the hypothesis that the loss of this cone type is related to the high scattering coefficients of very short-wavelengths of light ($\lambda < 450$ nm) in water (Kirk 1990). As noted by Lythgoe (1979), the reduction in sensitivity to these highly scattered wavelengths could serve to increase the visibility of broad spectrum or relatively long-wavelength visual targets. Similar hypotheses have been put forth to explain trends in short-wavelength cone sensitivity observed in fresh water cottoid fish (Bowmaker 1995). The associated loss of cone-based color vision is presumably an inadvertent consequence of the loss of S cone sensitivity, as it is even more difficult to imagine a scenario in which the ability to discriminate color might be detrimental to visual functioning.

Regardless of the selective mechanism underlying the loss, it is clear from the data obtained here that the cetaceans examined lack S cone visual pigments. The absence of this visual pigment type reduces the ability of these marine mammals to detect stimuli in the short-wavelength region of the spectrum and renders them incapable of making conventional cone-based color discriminations. Moreover, while the precise origin of the loss of this cone type is uncertain, it clearly occurred before the divergences of the mysticete and odontocete sub-orders. Consequently, by inheritance, all modern cetaceans are incapable of producing functional S cone visual pigments.

Acknowledgements

Hippopotamus DNA was graciously provided by the Center for the Reproduction of Endangered Species (CRES), San Diego, CA. Cetacean tissue samples were obtained from the cetacean tissue collection at the NMFS Southwest Fisheries Science Center in La Jolla, CA. Thanks to Drs. W. Perrin, R. LeDuc, G. Jacobs and two anonymous referees for thoughtful reviews of the manuscript, and to Dr. L. Galver, R. Tajrishi, C. LeDuc, K. Coutrip and M. Martinez for their assistance with data collection.

The text of this chapter, in part or in full, is a modified version of the material as it appears in Proceedings of the Royal Society of London - Series B: Biological Sciences **270**: 673-679. I was the primary researcher and/or author and the co-author listed in this publication directed and supervised the research which forms the basis for this chapter. Used here by permission.

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III. CHAPTER TWO

Photopic spectral sensitivity of green and loggerhead sea turtles

Abstract

Flicker electroretinography (ERG) was used to examine the *in situ* photopic (cone-photoreceptor based) spectral sensitivities of green and loggerhead sea turtles. Both species were responsive to wavelengths from 440 to 700 nm and both had peak sensitivity in the long-wavelength portion of the spectrum (~580 nm). For loggerhead turtles, no measurable responses were obtained below about 440 nm, while reliable signals were seen for green turtles at wavelengths down to 400 nm. Both species exhibited significant declines in sensitivity below 500 nm. The overall shapes of the spectral sensitivity functions were similar for the two species. These results support previous findings that sea turtles have well-developed photopic visual systems. The characteristics of these spectral sensitivity functions indicate that both species possess multiple cone photopigment types and these, in conjunction with the presence of colored oil droplets, strongly imply a capacity for color discrimination. Comparative evaluation suggests that these turtles have modified their visual pigments from those of their terrestrial relatives to better suit the ambient conditions present in the shallow water, submarine environments that they typically inhabit.

Introduction

Modern sea turtles diverged from their terrestrial and freshwater relatives roughly 150 million years ago (Pritchard 1997). They live an almost exclusively marine existence, returning to the ocean within a few hours of hatching, and subsequently rarely returning to land. Sea turtles are currently distributed worldwide throughout the tropical and sub-tropical oceans, with some species occasionally ranging into higher, more temperate latitudes. Sea turtles occupy a variety of ecologic niches. For instance, adult green turtles (*Chelonia mydas*) are generally found near shore feeding on plant and algal matter, while others, such as adult loggerhead turtles (*Caretta caretta*) may stay farther offshore, diving deeper to forage on benthic invertebrates and fish (Bjorndal 1997).

Anatomical examinations reveal that, in contrast to terrestrial and freshwater turtles, the eyes of sea turtles have round lenses, much like those found in fish, to compensate for the loss of corneal refraction under water (Walls 1942; Ehrenfeld and Koch 1967; Northmore and Granda 1991). The spectral sensitivity of marine turtles is also different from that of their land-based relatives (Liebman and Granda 1971; Granda and Dvorak 1977). Microspectrophotometric (MSP) evaluations of green turtle photoreceptors show that 11-*cis* retinal is used as the light sensitive chromophore component of sea turtle visual pigments; land-based turtles typically use 11-*cis* 3,4 di-dehydroretinal (Liebman and Granda 1971; Loew and Govardovski 2001). This substitution has the effect of substantially shifting the sensitivity of the longer wavelength sensitive visual pigments of marine turtles towards shorter wavelengths. MSP also reveals that the light absorbing oil droplets present in marine turtle cone photoreceptors are different from those of terrestrial and freshwater turtles (Granda and Haden 1970; Granda and Dvorak 1977).

Although there is evidence that the visual sense is important to sea turtles throughout all stages of their life cycle (Ehrenfeld and Carr 1967; Kingsmill and Mrosovsky 1982; Lohmann et al. 1990), relatively little is known about their *in situ* visual sensitivities; most previous work has been directed at the individual photoreceptors. As several conservation issues currently facing marine turtles can be related, at least in part, to the effects of anthropogenic light sources, there is a particular need for a better understanding of the visual abilities of living turtles (e.g., Witzell 1999; Witherington and Martin 2000). As a step in this direction we have used a noninvasive technique, rapid-flicker electroretinography (ERG), to investigate the photopic (cone-photoreceptor based) visual sensitivities of two marine turtle species, green and loggerhead sea turtles. This type of ERG is particularly relevant to behavioral/conservation issues, as it reflects the actual sensitivity of the eyes of the animal, including the combined effects of the cone visual pigments, oil droplets, and other sources of intraocular scattering and absorption (e.g. cornea, lens). Rapid-flicker ERG was chosen over the single flash ERG technique previously used with green turtles (Granda and O'Shea 1972), as it is better suited to isolating cone photoreceptor responses, and thereby potentially providing a more accurate representation of *in situ* photopic sensitivity. Furthermore, by conducting parallel experiments with two species we reasoned that since the individual cone photopigments of the green turtle have been previously measured, we could use the ERG measurements on green turtles to draw more precise inferences about the photoreceptor complements of loggerhead turtles.

Methods

Live adult (all greater than 30 years old) green and loggerhead sea turtles were examined at SeaWorld, San Diego, California. Recordings were obtained from four

green turtles and six loggerhead turtles. It was not possible to determine the gender of some of the turtles, but at least one male and one female were examined from each species. These animals were maintained in captivity in seawater exhibits before and after recording. All animal husbandry and experimental procedures were conducted according to protocols approved by the SeaWorld Institutional Animal Care and Use Committee (IACUC) and were overseen by the veterinary staff at SeaWorld.

Photopic (cone-photoreceptor based) spectral sensitivity was evaluated for each turtle *in vivo* using flicker electroretinography (ERG). For this examination animals were removed from their holding pools and anesthetized with intravenous administration of ketamine (6.0-9.0 mg/kg) and metatomadine (0.1 mg/kg) injected transdermally into the dilation of the external jugular vein at the base of the head. Once anaesthetized, the turtles were positioned on a slant board that was placed on an adjustable surgical table and the head position was stabilized through the use of padded restraints. Although the general anesthetic typically produced some degree of pupillary dilation, in some cases additional dilation was achieved by topical application of atropine and ophthalmic neosynephrine. The cornea was anesthetized by a topical application of proparacaine hydrochloride (0.5%) prior to the installation of a bipolar contact-lens electrode of the Burian-Allen configuration. After the experiments were completed, the effects of the anesthesia were partially reversed using atapamazole (0.1 mg/kg). In all cases full recovery was achieved without incident.

The general technique used to record flicker ERGs was that of Jacobs et al. (1996). In the present experiments the eye was stimulated with a train of light pulses originating from a high-intensity (50 W tungsten-halide lamp) grating monochromator having a half bandpass of 15 nm. The light was imaged onto the retina in Maxwellian

view in the form of a spot subtending 59 deg. The test light was temporally modulated with electromechanical shutters (Vincent Associates, Rochester, NY) as a square-wave pulse having a 25% duty cycle so as to achieve any desired flicker rate. The fundamental frequency component of the ERG response was extracted by filtering and this signal was averaged over a total of 50 presentations. Spectral sensitivity functions were measured using two techniques. In the first case a flicker photometric procedure was employed in which the responses to the test light and those given to an interleaved reference light (achromatic, 14 log photons/sec/sr) that illuminated the same region of the retina and flickered at the same temporal frequency were compared. Over successive presentations the intensity of the monochromatic test light was adjusted by changing the position of a 3.0 log unit neutral-density wedge until the light produced a response equal to that given to the fixed reference light. Repetition of this procedure for a range of different test wavelengths can be used to define a spectral sensitivity function (Jacobs and Neitz 1987). In a second set of measurements spectral sensitivity functions were similarly determined using a standard amplitude-criterion method. In this case the intensity of the test light at each wavelength was adjusted over successive presentations until it produced a response with constant amplitude of 3.2 μV .

ERG temporal response functions were also obtained. To accomplish this, the intensity of an achromatic (2850 K) flickering light was adjusted to produce a response having a criterion amplitude of 3.2 μV . This procedure was repeated for flickering lights varying in steps of 4 Hz from 4 Hz to 40 Hz. For all of the spectral sensitivity and rate measurements, thresholds were determined twice for each test condition and these values subsequently averaged. All recordings were made under moderate photopic illumination produced by a mixture of indirect skylight and

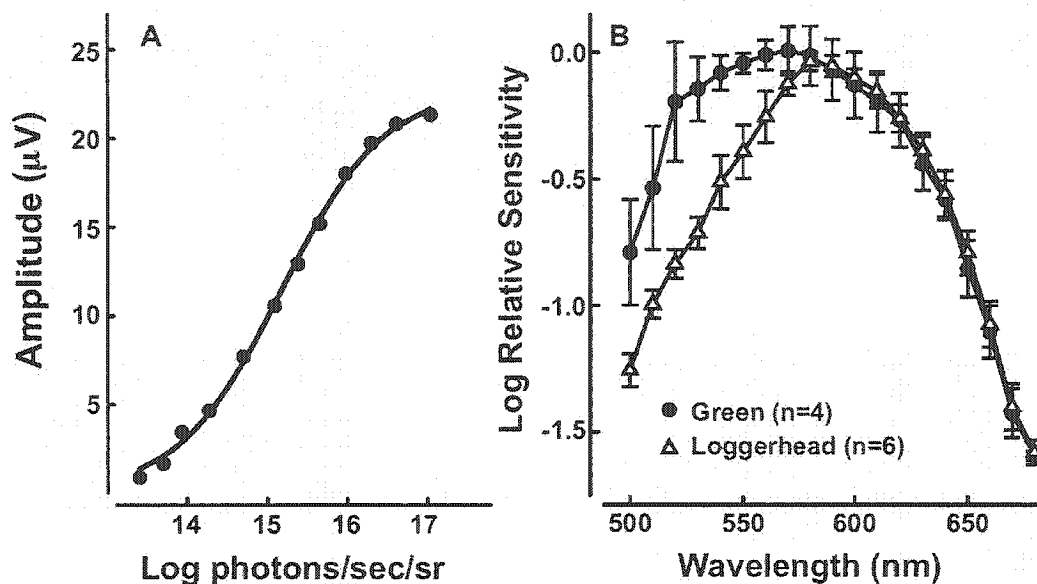


Figure 4. A. An example of a flicker ERG intensity/response function obtained from a Loggerhead sea turtle. The data points are mean amplitudes obtained from four presentations of an achromatic test light flickered at 20 Hz. The intensity of the stimulus is as specified at the cornea. The fitted line is a Michaelis-Menton function. The values of the three parameters for this fit are: $V_{\max} = 22.9 \mu\text{V}$; $k = 15.2 \text{ log/photons/sec/sr}$; $\eta = 0.68$. These three index, respectively, voltage at saturation, intensity required for half-maximum amplitude, and a slope parameter. B. Photopic spectral sensitivity curves for Green and Loggerhead sea turtles obtained with the flicker-photometric ERG procedure described in the text. The data points are mean values for the numbers of animals specified and the error bars represent ± 1 standard deviation. The data points have been interconnected with straight lines.

overhead fluorescent lighting that yielded an illuminance of 165 lux at the subject's eye.

Results

As recorded from the corneas of sea turtle eyes, flickering lights produced small but quite reliable ERG signals. For example, Figure 4A shows a representative intensity/response function obtained from a loggerhead turtle to 20 Hz flicker. The plotted points represent the mean amplitudes recorded for four successive presentations of the test light each of which in turn consisted of 50 stimulus cycles. The line fitted through the data points was derived from the Michaelis-Menton function, a metric typically used to account for intensity-response relationships for signals recorded early in visual systems, including photoreceptor responses recorded directly from cones of freshwater turtles (Granda and Dvorak 1977). Although the peak amplitudes recorded under these flickering-light test conditions were modest in size (in the range of 20-50 μV), they were, as noted, highly reliable; for example, the SD values for the responses of Figure 4A averaged less than 1 μV across the full span of stimulus intensity.

Figure 4B summarizes the spectral sensitivity functions obtained from 10 sea turtles as determined with ERG flicker photometry using 20 Hz flicker. The functions for both species peak at about 580 nm. The sensitivities of the two are indistinguishable from that point out to the longer wavelengths. However, the sensitivity curve for the loggerhead turtles falls off more steeply toward the short wavelengths than does the equivalent function obtained from the green turtles. With this technique it proved impossible to make sensitivity measurements for wavelengths shorter than about 500 nm. Note that the individual variability for each species over this spectral range is quite small with SD values averaging 0.1 log unit or less.

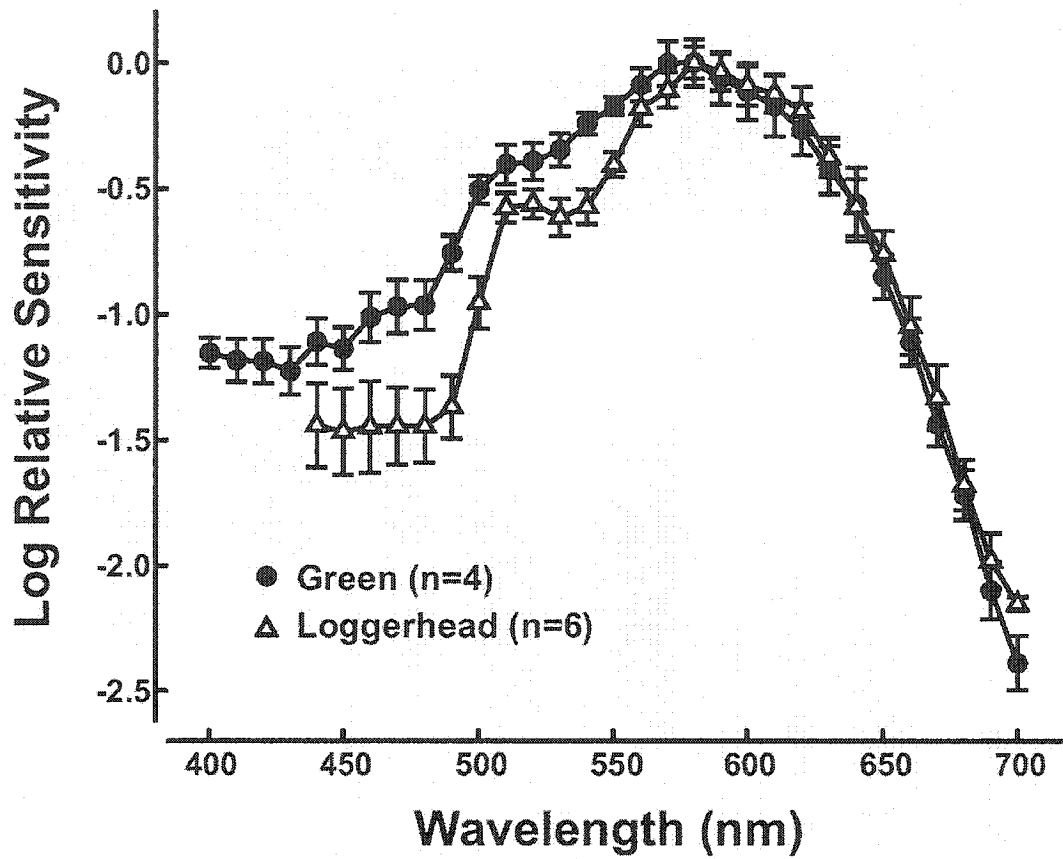


Figure 5. Photopic spectral sensitivity curves for Green and Loggerhead sea turtles obtained from an amplitude-criterion procedure. The test light was flickered at 20 Hz. Other details are the same as for Figure 4B.

In an attempt to extend the spectral range of the spectral sensitivity functions we also tested these same animals using the amplitude-criterion procedure. The results are shown in Figure 5, which again summarizes results obtained from the same 10 animals. With this procedure it proved possible to obtain sensitivity measurements from 700 nm well down into the short wavelengths (400 nm and 440 nm for the green and loggerhead turtles, respectively). As for the flicker photometric results, both species show a sensitivity peak at about 580 nm with a smooth (and equivalent) falloff in sensitivity to the longer wavelengths. To the shorter wavelengths the shapes of the functions for the two species are similar with a secondary peak at about 520 nm and a region of near constant sensitivity below about 500 nm. As in the flicker photometric spectral sensitivity functions, the loggerhead turtles had consistently lower sensitivity to all of the test wavelengths shorter than the principal peak. It is also apparent that the variability among animals is larger for the shorter test wavelengths and that this is particularly marked for the loggerhead turtles at wavelengths shorter than 500 nm. Variability within individuals remained relatively consistent across the spectral range examined.

All of the above measurements were made at a single temporal frequency (20 Hz). To examine of the effects of temporal rate on sea turtle ERGs, we also measured thresholds for achromatic lights across a range of temporal frequencies. For both species temporal sensitivity was maximal over a relatively broad range extending from about 8-16 Hz. Sensitivity at 4 Hz was only 0.25 to 0.5 log units below peak sensitivity. At flicker rates above 12-16 Hz, sensitivity gradually fell off to up to 36 Hz, where thresholds for both species were over 2.0 log units below peak values. At 40 Hz, no consistently reliable responses could be recorded and straight-line extrapolation of the frequency/sensitivity curve suggests that 40 Hz represents the

high-frequency cutoff for both turtle species. For frequencies greater than the peak, the sensitivity of the loggerhead turtles was consistently (but not dramatically) lower than for the green turtles. This presumably reflects the lower sensitivity of the former species to shorter test wavelengths.

Discussion

Previous measurements of the spectral sensitivity of green turtles have been made using a single-flash ERG technique (Granda and O'Shea 1972). In both the dark- and light-adapted eye the spectral sensitivity functions had multiple peaks at about 600 nm, 520 nm, and 460 nm. The spectral sensitivities we measured for green turtles are roughly similar to these earlier results, although the long wavelength peak is somewhat shorter in the present measurements ($\lambda_{\max} \sim 580$ nm) and the short-wavelength peak is much less clearly defined. There are other differences between the two experiments, two of which are potentially important. The first is that the use of high frequency flicker ERG more securely isolates signals from cones than the single-flash procedure, even when the latter is conducted in a light-adapted eye. It is thus conceivable that some contributions from rods are represented in the earlier study's spectral sensitivity results. The relatively high frequency temporal responses of both species (up to 36 Hz) support the conclusion that our data are representative of cone photoreceptor responses (see Jacobs et al. 1996). Perhaps more important is the great difference in age of the turtles examined in the two experiments. In the earlier experiment the turtles were very young (somewhere between two and sixteen months of age) (Granda and O'Shea 1972). There are not sufficient records available to age our subjects accurately. The staff at SeaWorld indicates that they are all at least 30 years of age, and perhaps some turtles are substantially beyond that point. It is conceivable that with age the ocular components of sea turtle eyes (cornea, lens, etc)

become considerably less transmissive. In humans where age-related losses of ocular transmissivity have been extensively studied these changes are often found to differentially filter out short wavelength ($\lambda < 500$ nm) energy (e. g., Weale 1988). If this is also true for sea turtles it might explain the differences in short wavelength sensitivity seen in the two studies. In any case, the results of the two studies suggest there may be differences in short-wavelength sensitivity between young and aged sea turtles.

Earlier MSP measurements revealed the presence of four types of photopigment in the retina of the green turtle: a rod pigment with a 502 nm peak and three types of cone pigment with respective peak values (λ_{\max}) of 440, 502 and 562 nm (Liebman and Granda 1971). As is typical of many reptiles, as well as a number of birds, amphibians, monotremes and fishes, the cone photoreceptors of the green turtle also contain high-density oil droplets (Granda and Haden 1970). Since these oil droplets are interposed in the optical pathway to the outer segments, if colored, they can serve to spectrally filter incoming light. In the green turtle there are three classes of oil droplets, clear, yellow and orange. They are selectively paired with the cone pigments to form what are potentially six spectrally discrete classes of receptor (Liebman and Granda 1971). The net effect of these pairings is to effectively shift the spectral sensitivity of two of the cone classes to longer wavelengths. As in Granda and O'Shea (1972), the shifted positions of the two longer wavelength peaks in the green turtle ERG sensitivity curve reflect this (Figure 5).

The spectral sensitivity functions obtained from loggerhead turtles are similar in shape to those derived for green turtles suggesting a similar cone pigment and oil droplet complement in the two species. Direct comparisons can be made for one of the cone pigments in the two species. This is because, as noted above, the oil droplets

function as long-pass filters. The longest of these, the yellow droplets, show no detectable absorption for wavelengths longer than about 600 nm (Liebman and Granda 1971). Consequently, the long wavelength limb of the spectral sensitivity functions should reflect principally the absorption properties of the longest of the cone pigments. To determine the spectral position of this pigment we shifted a standard photopigment absorption function (Govardovskii et al. 2001) along the wavelength axis to determine what pigment position best accounted for the array of sensitivity values for test lights of all wavelengths 600 nm and longer. For the two estimates of sensitivity obtained for green turtles (Figures 4B and 5), these peaks were at 563 and 559 nm respectively. These values are very close to the position of the cone pigment directly measured with MSP (562 nm). A similar test of the two spectral sensitivities obtained from loggerhead turtles yielded estimates of 562 and 563 nm. We conclude that these two species have in common their longest cone pigment. Given the uncertainties produced by oil droplet/pigment combinations described above, a similar comparison of other cone pigment positions is not possible, although the general similarity of the shapes of the spectral sensitivity functions strongly suggest that they too are the same for these two sea turtles.

In general, the retinas of most turtles (Walls 1942; Granda and Dvorak 1977), including the marine species (Granda and Dvorak 1977; Bartol and Musick 2001) are cone dominated, with cone:rod photoreceptor ratios of 2:1 or more (Bartol and Musick 2001). This is unlike other diving tetrapods, such as marine mammals that typically have large, rod dominated eyes to maximize light capture, and a limited range of spectral sensitivity (Lavigne et al. 1977; Peichl et al. 2001; Levenson and Dizon 2003). Similarly, many fish, particularly those inhabiting depths visited by the deeper diving turtles (>100 m), are strongly adapted to maximize light sensitivity, and exhibit

evidence of reduced photopic visual abilities (Lythgoe 1979; Bowmaker 1995). This often includes a relatively narrow range of spectral sensitivity in comparison to similar, shallow water species (Bowmaker et al. 1994; Lythgoe et al. 1994). Qualitative evaluation of sea turtle visual systems suggests that these animals have largely retained diurnally adapted visual systems like those of their reptilian/avian relatives. Their broad spectral sensitivity corresponds well with the type of light available in relatively shallow, well-lit marine waters, rather than that of the dim, nearly monochromatic deep oceanic water (Kirk 1994). They also have relatively small lenses (Northmore and Granda 1991) and cone dominated retinae with additional light filtering oil droplets (Granda and Haden 1970; Bartol and Musick 2001). Even the very deep-diving leatherback sea turtle (Eckert et al. 1989) does not possess a particularly large lens, which might be expected if these turtles were adapted for maximizing light capture (Northmore and Granda 1991).

The presence of multiple functioning photoreceptor types also suggests that these turtles have retained the ability to make color discriminations. Such abilities are lost in sensitivity-adapted marine mammals (Peichl et al. 2001; Levenson and Dizon 2003) and fish from deeper water habitats (e.g., Bowmaker et al. 1994; Cowing et al. 2002), but are relatively common in diurnally adapted fish, birds and other reptiles (Yokoyama and Yokoyama 1996; Bowmaker 1995). Color vision and the retinal circuitry that supports that capacity have been extensively studied in freshwater turtles. Evidence suggests that in these turtles the array of pigment and oil droplet combinations and the neural support provide a multidimensional color vision system that is probably at least tetrachromatic in nature (Neumeyer 1998). Although both cone pigments and oil droplets differ for freshwater and sea turtles, the presence of multiple pigments and oil droplets in sea turtles strongly suggests that they are like

their freshwater cousins in enjoying a color vision capacity. The nature of that capacity remains to be determined.

All these observations suggest that the vision of sea turtles is primarily suited for use in photopic conditions. There is, however, also reason to conclude that the visual systems of sea turtles have been modified for use in the marine environment. In comparison to closely related terrestrial turtles, both species of sea turtle exhibited long wavelength sensitivity indicative of visual pigments that have been shifted towards the shorter wavelengths of light ($\lambda \leq 500$ nm) typically prevalent in marine environments (Kirk 1994). The cone pigments of the green and likely those of the loggerhead turtle have λ_{\max} 18 to 55 nm shorter in sensitivity than those of the land-based red-eared slider (*Trachemys scripta elegans*; Liebman and Granda 1971; Loew and Govardovskii 2001). Much of this sensitivity change is undoubtedly related to a shift in chromophore. The essentially identical long-wavelength sensitive pigments of green and loggerhead turtles suggest that both species, and perhaps then all marine turtles, have made the chromophore substitution to retinal from 3,4 di-dehydroretinal (Granda and Dvorak 1977). This same disparity is common between marine and freshwater fish species; marine fishes typically have retinal in their visual pigments and the corresponding short wavelength shift in sensitivity (Bowmaker 1995).

The conclusion that sea turtle visual pigments are well suited for use in relatively well-lit, shallow-water marine environments concurs with the observed diving/foraging behavior of most species (Bjorndal 1997). The photopic spectral sensitivities of these sea turtles are clearly different from those of the other diving tetrapod groups, as are they different from their non-marine relatives. In fact, the cone visual pigments of sea turtles are convergent in many ways with those of some of the tropical, shallow-water marine fish with which they share habitat. Both have multiple

pigment types and a broad range of sensitivity, but are short-wavelength shifted in sensitivity when compared to similar species from freshwater/terrestrial habitats (Lythgoe et al. 1994; Bowmaker 1995; Losey et al. 2003). Indeed, considering the absence of reductions in photopic function for increased overall sensitivity seen in deep-water fish and mammals, one might speculate that the visual sense may not be of great consequence to deep-diving loggerhead and leatherback turtles when they venture to depths in excess of a few hundred meters, as they sometimes do (Eckert et al. 1989; Bjorndal 1997). This remains to be determined.

However, it is also important to consider the ecological and behavioral ramifications of visual stimuli when considering the significance of the sensitivity data presented here. The results from ERG are strictly a representation of the sensitivity of the eyes, and do not reflect the behavioral significance that visual stimuli may contain. For example, though less sensitive to short wavelength lights, both green and loggerhead turtles will reliably orient towards shorter wavelength stimuli and away from long wavelength lights when given a choice (Witherington and Bjorndal 1991). This behavior, which is thought to help hatching turtles re-enter the ocean, is contradictory to what might be predicted given only the measured spectral sensitivities of these species. Thus, photoreceptor sensitivity should only be one of many factors to be considered when examining the behavior of sea turtles or working to mitigate the effects of human activities on sea turtle populations.

Acknowledgements

Thanks to SeaWorld, San Diego for their willingness to allow us to examine their animals, and to the SeaWorld animal husbandry staff for their skilled assistance in all aspects of the experiments (IACUC permit RR2001-15). Thanks also to Drs. W. Perrin, A. Dizon, P. Ponganis, G. Kooyman, and several anonymous reviewers for critical reviews of earlier versions of the manuscript. This work was supported by a grant from the National Marine Fisheries Service, Honolulu, HI to D. Levenson and S. Eckert.

The text of this chapter has been submitted for publication in the journal *Copeia*. I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

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IV. CHAPTER THREE

Genetic and electroretinographic examination of pinniped visual pigments, with comments on the polar bear and the sea otter

Abstract

Pinnipeds (seals, sea lions, and walrus) are amphibious mammals. The rod and cone visual pigments of seven pinniped species were evaluated to examine how these mammals have adapted to visual conditions inherent to their amphibious lifestyle. Species from all three pinniped families were represented. Additionally, the polar bear (*Ursus maritimus*) and the sea otter (*Enhydra lutris*) were examined for comparative purposes. Molecular genetic techniques were used to obtain rod opsin, middle/long-wavelength sensitive (M/L) cone opsin and (when present) short-wavelength sensitive (S) cone opsin sequences from retinal messenger RNA. Spectral sensitivity was subsequently inferred from transcribed opsin nucleotide sequence data through the evaluation of known spectral tuning residues. The rod pigments of nearly all of the pinnipeds were similar to those of the sea otter, the polar bear, and other terrestrial carnivores with maximum sensitivities (λ_{\max}) of 499 or 501 nm. Only the rod opsin sequence of the northern elephant seal (*Mirounga angustirostris*) had sensitivity characteristic of adaptation for vision in the marine environment, with an inferred λ_{\max} of 487 nm. The M/L cones of the pinnipeds examined were also similar to those of terrestrial carnivores, with inferred λ_{\max} of 550-560 nm. No evidence of S cones was found for any of the pinnipeds. The polar bear and the sea otter had S cones similar to those of other terrestrial carnivores.

Flicker-photometric electroretinography (ERG) was used to examine the *in situ* spectral sensitivities of a subset of these pinniped species: northern elephant seal, harbor seal (*Phoca vitulina*), and California sea lion (*Zalophus californianus*). Recordings were made under conditions previously shown to elicit cone responses in a wide range of other mammals. Remarkably, no responses could be elicited from the M/L cones of any species, nor was there any indication of S cone activity. All three

pinnipeds exhibited sensitivities closely resembling those of their respective rod pigments. The nature of the disparity between ERG and genetic results is discussed. In summary, the visual pigment complement of pinnipeds directly reflects their amphibious lifestyle: their rod and M/L cone sensitivities are generally similar to those of terrestrial carnivores but, like the completely aquatic cetaceans, are lacking S cones.

Introduction

Pinnipeds are unique among mammals in that they are the only large monophyletic lineage that is distinctly amphibious. The pinniped group is composed of more than 30 species from three families: Otariidae (fur seals and sea lions), Odobenidae (walrus), and Phocidae (true seals). Fossil evidence indicates that pinnipeds began their re-invasion of the marine environment between 28 and 35 million years ago (MYA; Berta and Summich 1999). Pinnipeds forage exclusively in the water but must return to land to give birth to their young. This makes them particularly interesting from an evolutionary standpoint in that they represent a decisive intermediate stage between terrestrial and aquatic adaptation, and they have had a significant amount of time to evolve into their amphibious lifestyle.

Vision is important to pinnipeds both on land and in the water, and there is some evidence that their visual systems have become distinct from those of their terrestrial ancestors to function more effectively in these two diverse media (Walls 1942). One aspect of the visual system that may provide a good index for evaluating the nature and extent of the amphibious adaptation of pinnipeds is their visual pigments. The visual pigment complement of a species is often closely correlated to its visual environment, owing at least in part to the plasticity of these pigments (Jacobs 1993; Bowmaker 1998). This has been found to be particularly true in the marine environment where visual conditions vary dramatically (Bowmaker 1995; Hunt et al. 1996; Fasick and Robinson 2000). Visual pigments are apoproteins, composed of a light sensitive chromophore, 11-*cis* retinal, attached to a protein moiety, opsin (Wald 1968). Among mammals, three classes of visual pigment are typically present in the retina, each characterized by its underlying opsin protein: a rod opsin found in the rod photoreceptors, and two opsin types found in the cone photoreceptors, the short-

wavelength (S) and middle/long-wavelength (M/L) sensitive cone opsins (Yokoyama and Yokoyama 1996). While only three opsin classes exist, minor changes to the primary structures of individual opsins within these classes can tune the spectral sensitivity of the corresponding visual pigments over a wide range of wavelengths. In addition, the underlying genetic changes to the opsin proteins that affect this spectral tuning are now relatively well understood, making it possible in many cases to infer the spectral sensitivity of a visual pigment directly from the genetic sequence of its opsin protein (Fasick and Robinson 2000; Yokoyama and Radlwimmer 2001; Shi and Yokoyama 2003).

Some evidence already exists that the visual pigments of pinnipeds are different from those of their terrestrial relatives. Although some of these animals have maintained rod photoreceptor pigments similar to those of terrestrial mammals (Cresticelli 1958; Lavigne and Ronald 1975; Fasick and Robinson 2000), some deep-diving species have modified scotopic (rod photoreceptor) visual pigments that provide increased sensitivity to the blue-green wavelengths of light ($\lambda \sim 475$ nm; Kirk 1994) that predominate in deep ocean water (Lythgoe and Dartnall 1970; Southall et al. 2002). There is also evidence that the photopic (cone photoreceptor) visual pigments of some pinnipeds have been modified for use in the marine environment. Although most mammals possess two distinct cone pigment types (Jacobs 1993; Yokoyama and Yokoyama 1996), several pinniped species have been found to possess only M/L cones (Peichl and Moutairou 1998; Peichl et al. 2001, 2002). At present, the only large group of mammals known to completely lack S cones is the entirely aquatic cetaceans (Levenson and Dizon 2003). The M/L cones that are present in pinniped retinæ may also have been modified for use under water, though only a few observations have been made. The M/L cones of terrestrial carnivores typically have

maximum sensitivity of 545-560 nm (Jacobs et al. 1993; Yokoyama and Radlwimmer 1998, 1999). Yet, in ERG experiments, the harbor seal (*Phoca vitulina*) exhibited maximum spectral sensitivity near 510 nm (Crognale et al. 1998). A behavioral evaluation of the harp seal (*Pagophilus groenlandicus*) also reported a peak in spectral sensitivity between 500 and 525 nm, as well as a second peak near 550 nm (Lavigne and Ronald 1972; Lavigne et al. 1977).

Although there is some evidence of adaptation for aquatic vision in the rod and cone visual pigments of pinnipeds, further evaluation of additional species with specific data about individual pigments is necessary to fully understand the nature of their amphibious adaptation. Towards this end, molecular genetic methods have been used to examine the opsin proteins underlying the rod, S cone, and M/L cone photoreceptor pigments of species from all three pinniped families and representing several different ecologic niches. For comparative purposes, the sea otter (*Enhydra lutris*) and the polar bear (*Ursus maritimus*) were also examined, along with opsin sequences obtained from Genbank. Flicker-photometric electroretinography (ERG) was additionally used to compare the *in situ* spectral sensitivities of some of these pinniped species with the sensitivities of their visual pigments as predicted from the genetic data.

Methods

GENETIC EVALUATION OF ROD AND CONE VISUAL PIGMENTS

To evaluate the visual pigments expressed in the retinas of marine carnivores, rod and cone opsin sequence data was obtained from retinal messenger RNA (mRNA). The polar bear, the sea otter, and seven pinniped species were examined (Table 2). Here forward pinnipeds will be referred to by their binomial scientific name (i.e., *Genus species*) and all other mammals by their common names. Retinae from an

Table 2. Summary of the carnivore species (Carnivora) examined. Uncorrected "p" distances are shown for each gene as compared to the cat (rod, M/L cone) and cow (S cone). Taxon names after Rice (1998). * Genetic data from Fasiak and Robinson (2000). # Data from Genbank.

Family	scientific name	common name	N	rod opsin	Uncorrected "p" distance M/L cone opsin	S cone opsin
Mustelidae	<i>Enhydra lutris</i>	sea otter	2	0.07684	0.08403	0.10527
Ursidae	<i>Ursus maritimus</i>	polar bear	1	0.06375	0.07587	0.10623
Phocidae	<i>Pagophilus groenlandicus</i>	harp seal	-	0.0659 *	0.0895 #	not examined
Phocidae	<i>Phoca vitulina</i>	harbor seal	2	0.06686 *	0.09132 #	none found
Phocidae	<i>Pusa hispida</i>	ringed seal	2	0.06458	0.08652	none found
Phocidae	<i>Erignathus barbatus</i>	bearded seal	1	0.0698	0.08399	none found
Phocidae	<i>Mirounga angustirostris</i>	Northern elephant seal	3	0.06479	0.08922	none found
Phocidae	<i>Leptonychotes weddellii</i>	Weadell seal	2	0.06972	0.08674	none found
Phocidae	<i>Hydrurga leptonyx</i>	leopard seal	1	0.08133	0.08354	none found
Otariidae	<i>Zalophus californianus</i>	California sea lion	2	0.06163	0.07964	none found
Odobenidae	<i>Odobenus rosmarus</i>	walrus	2	0.0629	0.0806	none found

additional species, *Phoca vitulina*, were also examined for the presence of S cones. In addition, published M/L sequences for *P. vitulina* and *Pagophilus groenlandicus* were obtained for evaluation of spectral sensitivity.

Eyes used for molecular analysis were obtained from animals that had died of natural causes or were euthanized in captivity or wildlife rehabilitation facilities as a consequence of terminal illness/injury. Samples were collected as soon as possible after death and subsequently kept frozen at -20 °C or colder. To obtain retinal mRNA, each eye was thawed until the retina could be removed. The excised retinal tissue was then homogenized in Trizol (Gibco BRL, Rockville, MD; ~1 ml/100 mg of tissue) and the RNA extracted and precipitated with chloroform and isopropanol. After extraction, samples were incubated in DNase I (Ambion, Austin, TX) at 37 °C for 45 min to eliminate any DNA contamination. The purified mRNA was then converted to cDNA by incubating ~50 ng with MMLV reverse-transcriptase (Clontech/BD Biosciences, Palo Alto, CA), oligonucleotide (dt)₁₈ primer, and excess dNTP's for 60 min at 42 °C. Non-retinal, nuclear DNA was obtained for *Phoca vitulina*, *Mirounga angustirostris*, and *Zalophus californianus* from skin using a lithium-chloride extraction and ethanol precipitation procedure (Hillis et al. 1996).

Rod and cone opsin gene sequences were amplified and sequenced from the cDNA using polymerase chain-reaction (PCR) and dideoxy-terminator cycle sequencing (Sanger et al. 1977). An attempt was made to amplify and sequence rod, M/L and S cone opsins from the retinal mRNA/cDNA of every individual examined. Oligonucleotide primers for PCR and sequencing were 15-23 base pairs (bp) in length and were designed from homologous gene sequences of cow, human, mouse, dog, cat, and harbor seal obtained from Genbank. PCR primers for the S cone opsin were additionally optimized for marine carnivores using the *P. vitulina*, *M. angustirostris*,

and *Z. californianus* sequences obtained from nuclear DNA. For each opsin gene, two sets of forward and reverse primers were used to obtain two overlapping 400-650 bp sequence fragments. Primer annealing locations were chosen so that the consensus of the two fragments would provide sequence data for nearly the entire gene, including all currently described spectral tuning sites for each opsin type.

Each 50 μ l PCR reaction consisted of 5 μ l of cDNA (~ 100 ng), 36 μ l of water, 5 μ l of 10X PCR buffer with $MgCl_2$ (2.0 mM), 1 μ l of each oligonucleotide primer (10 μ M), and 1 μ l of *Taq* DNA polymerase. For amplification, reactions were initially denatured for 5 min at 94 °C and then cycled 35 times through the following conditions: denature for 45 s at 94 °C, anneal for 1 min at 55 °C, and extend for 1.5 min at 72 °C. Cycling was followed by a final 5 min extension at 72 °C. PCR products were analyzed qualitatively and by size using ethidium-bromide stained agarose-gel electrophoresis. If a reaction failed to produce a desired product, the annealing temperature of the PCR was adjusted to between 37 and 65 °C to improve primer performance. This procedure was repeated as necessary until the product was obtained or until the entire range of temperatures (in 1° increments) had been attempted. Once a desired fragment was amplified, the PCR product was purified using Qiaquick columns (Qiagen, Valencia, CA), cycle-sequenced using Applied Biosystems (ABI) Big Dye Version 3 chemistry (Applied Biosystems, La Jolla, CA), and analyzed with an automated ABI 3100 capillary sequencer. The obtained sequences were aligned with homologous sequences of other select mammals to evaluate their identity and the condition of known spectral tuning sites. Alignments were performed with ClustalW (Thompson et al. 1994) and confirmed by eye. Phylogenetic trees and nucleotide identity proportions were determined using PAUP software (Swofford 2001). Spectral sensitivities were inferred for each opsin

according to available information. These procedures varied for each opsin type and are described below.

ELECTRORETINOGRAM MEASUREMENTS

Visual pigments were evaluated *in situ* using electroretinograms (ERGs) for a subset of the pinniped species examined with genetics. Juvenile pinnipeds were obtained from the marine mammal rehabilitation program at SeaWorld, San Diego, CA. Included in this group were one female and three male *Z. californianus*, one male *P. vitulina*, and two female *M. angustirostris*. The animals were maintained in seawater tanks at the Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, La Jolla, CA, prior to recording and then until their eventual release to the wild. All animal husbandry and experimental procedures were conducted following protocols approved by the National Marine Fisheries Service (NMFS) Office of Protected Resources (permit 732-1487) and the University of California, San Diego Institutional Animal Care and Use Committee (IACUC; permit S00092).

Spectral sensitivity measurements were made using ERG flicker-photometry. The general technique of Jacobs et al. (1996) was used here. In the current application animals were masked and initially anesthetized with 5% isoflurane or halothane. After induction of anesthesia they were intubated and maintained on 0.75-2.0% isoflurane/halothane for the remainder of the experiments. The electrocardiogram and heart rate were monitored throughout testing. As in earlier measurements made on marine mammals (Crognale et al. 1998), anesthetized animals were placed on a slant board and the head position was stabilized through the use of padded restraints. The cornea was anesthetized with topical application of proparacaine hydrochloride (0.5%). Atropine (0.04%) and ophthalmic neosynepherine (10%) were then applied

topically to achieve pupillary dilation. ERG's were sensed with bipolar contact-lens electrodes of the Burian-Allen design.

In the flicker photometric procedure, the eye is stimulated with a train of light pulses that originate from two sources: a high-intensity grating monochromator having a 10 nm passband (the test light) and an achromatic reference light drawn from a tungsten-halogen lamp (the reference light). The two lights are each modulated with a 25% duty cycle. The fundamental components of the ERGs elicited by the two lights are extracted by filtering, averaged over 50 presentations, and then compared. The intensity of the test light is adjusted until the response it elicits exactly matches that produced by the reference light (Jacobs et al. 1996). The intensity of the test light at this point of equation is taken as a measure of the relative sensitivity of the eye to that test light. During the course of any experiment, each equation point is determined on at least two occasions and these values subsequently averaged. For these experiments, deviations between matches typically did not exceed 0.04 log units. The frequency of the flickering lights, the wavelengths of the test lights, and the intensity of the reference light were varied across experiments and these are noted below as they are appropriate.

Results

GENETIC SEQUENCING OF ROD AND CONE OPSINS

Putative rod and M/L cone opsin sequences were obtained from retinal mRNA/cDNA for all species examined. Both rod and M/L cone nucleotide sequences exhibited genetic distances of less than 0.1 from homologous opsin sequences of their terrestrial relatives (Table 2). Putative S cone sequences obtained for the sea otter and polar bear showed similarly high levels of identity with other mammalian homologues. No introns or mis-sense mutations were apparent in any of these

sequences. No S cone opsin sequences could be obtained from the retinal mRNA/cDNA of any of the pinnipeds examined. All eight species, including *Phoca vitulina*, were tested over a broad range of annealing temperatures, 37 to 65 °C, using the same primers that worked effectively on the polar bear and sea otter. Mg²⁺ conditions were also varied from 1.5 – 3.0 mM in a further attempt to obtain S cone opsin product without success. As a positive control of the oligonucleotide primers, one of the S cone primer sets was used to amplify a fragment of S cone opsin from the nuclear DNA of *P. vitulina*, *M. angustirostris*, and *Z. californianus*. Sequences were readily obtained and the exon regions of these nuclear DNA sequences showed similarly high levels of identity with other S opsins. However, these sequences were easily distinguishable from those of the polar bear and sea otter that originated from retinal mRNA/cDNA by the presence of a ~300 bp internal intron sequence.

Figure 6 shows the results of a parsimonious bootstrap analysis of the opsin sequences obtained from retinal mRNA. Also included are several sequences from Genbank. All three opsin genes grouped exclusively with their respective homologues. Both the rod and M/L cone opsin gene trees represent all species examined in this study and are nearly identical in structure. The M/L tree has slightly better resolution, presumably as a result of increased sequence variability (Table 2). Both trees identify the pinnipeds as a monophyletic group. In the M/L tree, the family Ursidae is indicated as the closest relative to the pinnipeds, with the mustelid sea otter as the outgroup among these carnivores. Within the pinnipeds, the families Odobenidae and Otariidae grouped together as sister clade to the phocid pinnipeds. In both gene trees the phocids are grouped into two distinct groups: one contains all the northern hemisphere species except *M. angustirostris*, the other contains all the southern species and *M. angustirostris*.

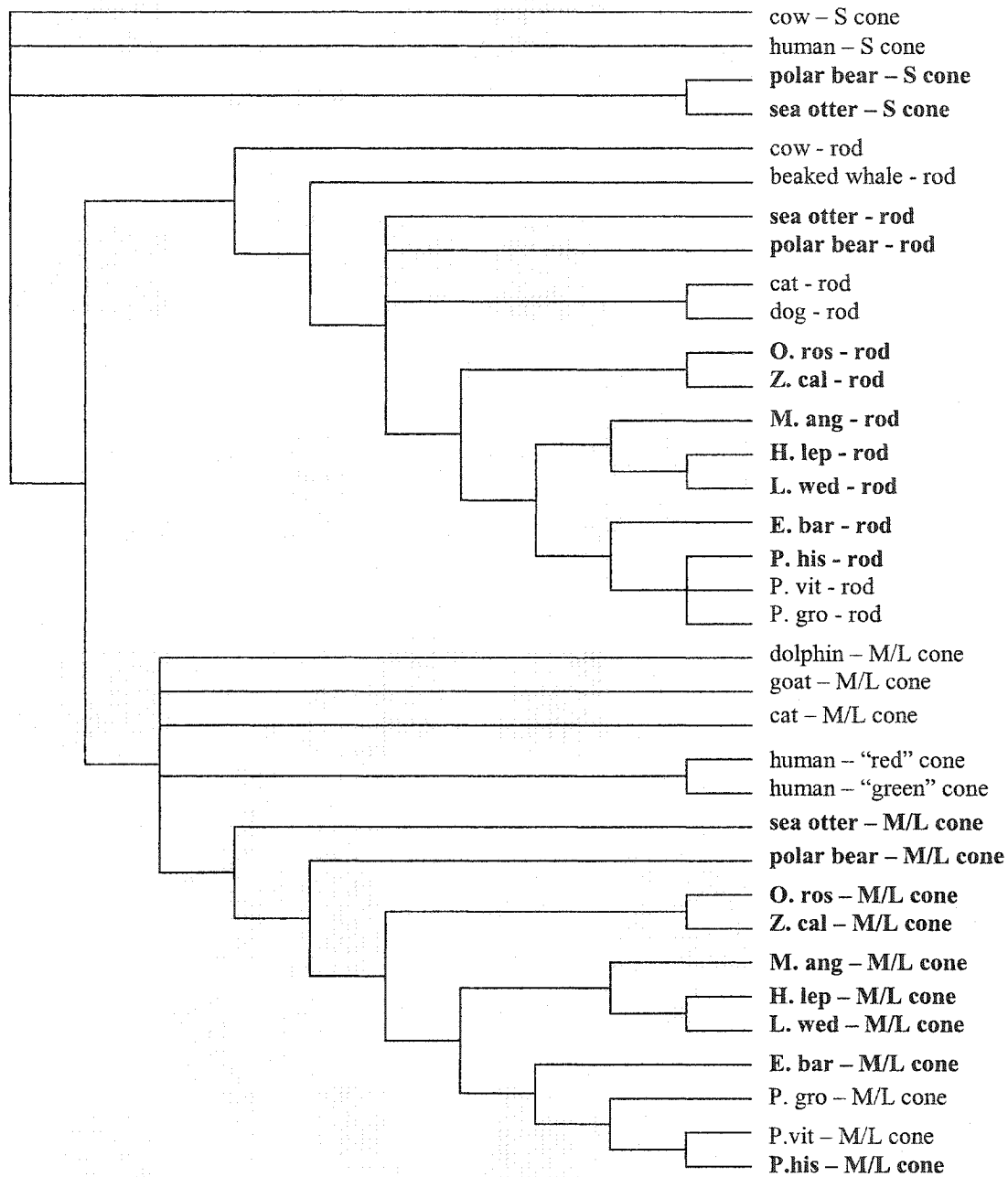


Figure 6. Parsimonious phylogenetic tree of rod, M/L, and S cone opsin sequences created using bootstrapping (1000 replicates). Pinnipeds are listed by abbreviated forms of their binomial designation (see Table 2), followed by the opsin type. Bootstrapping values above 50 were retained. Opsins from this study are shown in bold.

	10	20	30	40	50	60	70	80	90
dog	MNGTBCGNFY	VPFSNKTGVV	RSPFEYPQYY	LAEPWQFSML	AAYPELLIVL	GFPINFLITLY	VTQHKKLR	PLNYILNL	VADLFMVFVG
cat
P.gro
P.vitF.....
P.hisF.....
E.barF.....M.....I.....
M.angF.....I.....
L.wedF.....
H.lepF.....
Z.calH.....F.....N.....
O.rosS.....N.....
p.bearS.....
otterH.....I.....L.....
cowA.....M.....
whaleL.....H.....V.....M.....N.....L.....
	100	110	120	130	140	150	160	170	180
dog	FTTTLVTSLSH	GYFVFGPTGC	NVEGPFATLG	GEIALWSLVV	LAIERYVVVC	KMSNFRPGE	NHAIMGVAFT	WVMALACAAP	PLAGWSRYIP
catL.....V.....
P.groL.....GL.....V.....
P.vitL.....G.....V.....
P.hisL.....G.....V.....
E.barL.....V.....
M.angL.....L.....F.....V.....
L.wedI.....L.....L.....
H.lepI.....L.....
Z.calV.....L.....M.....V.....
O.rosA.....L.....V.....
p.bearL.....V.....
otterV.....L.....V.....
cowL.....V.....
whaleM.....	A.....I.....L.....L.....	L.....I.....V.....
	190	200	210	220	230	240	250	260	270
dog	EGMQSCGID	YITLKEINN	ESFVIYMPVV	HFAIPMIVIF	FCYQLVPTV	KEAAAQQQES	ATTQKAEKEV	TRMVIIMVIA	PLICWVPYAS
catV.....T.....
P.groV.....T.....
P.vitV.....T.....
P.hisV.....T.....
E.barV.....T.....A.....
M.angL.....V.....T.....T.....
L.wedL.....V.....T.....T.....V.....
H.lepL.....V.....T.....T.....V.....
Z.calV.....T.....
O.rosV.....T.....
p.bearV.....T.....L.....G.....
otterN.....V.....T.....L.....G.....
cowPHE.T.....I.....L.....L.....G.....
whaleV.....PS.....V.....V.....S.....VI.....V.....V.....
	280	290	300	310	320	330	340		
dog	VAFYIFTHQG	SDFGPIFMTL	PAFPAKSSSI	YNPVIYIMMN	KQPRNCMITT	LCCGKNPLGD	DEASASAKT	ETSQVAPA*	
catN.....L.....TTG.....*	
P.groFN.....AAA.....T.....V.....*	
P.vitN.....AA.....T.....V.....*	
P.hisN.....AA.....T.....V.....*	
E.barN.....GA.....T.....V.....*	
M.angN.....I.....S.....AA.....I.....*	
L.wedN.....T.....AA.....V.....*	
H.lepN.....T.....AA.....V.....*	
Z.calN.....I.....V.....*	
O.rosN.....I.....V.....*	
p.bearN.....*	
otterN.....I.....T.....T.....*	
cowN.....I.....T.....AV.....V.....TTV.....*	
whaleN.....I.....S.....A.....L.....R.....V.....TT.....*	

Figure 7. Alignment of the obtained rod opsin sequences, as well as those of two dog, cat, cow, deep-diving beaked whale, and two previously examined pinnipeds, *Pagophilus groenlandicus* and *Phoca vitulina* (Fasick and Robinson 2000). Pinnipeds are listed by abbreviated forms of their binomial designation (see Table 2), other species by their common name. Dots (.) indicate identity with the canine sequence. Dashes (-) indicate gaps or missing data.

A translated alignment of the obtained rod opsin sequences is shown in Figure 7. Rod opsin sequences of two terrestrial carnivores, dog (*Canis domesticus*) and cat (*Felis catus*), as well as the cow (*Bos taurus*) and a deep-diving beaked whale (*Mesoplodon bidens*) are included for comparison. Table 3 summarizes the 23 amino acid differences that exist between the pinniped rod opsins and those of their terrestrial carnivore relatives. Using the list compiled by Hunt et al. (2001) from a combination of previous mutagenesis studies and the 3-dimensional crystal structure of frog rhodopsin (Schertler and Hargrave 1995), all residues that could potentially affect the spectral sensitivity of the rod opsins were identified. As indicated in Table 3, residues 83, 292, and 299 (numbered from the bovine rod opsin) were variable and are known to be spectrally significant. Consequently, these were used in the inference of spectral sensitivity. Remarkably, not one of the 20 other variable positions occurred at sites of potential spectral significance as defined by Hunt et al. (2001). In fact, eleven of these were synonymous amino acid substitutions (i.e., polar amino acid for polar amino acid, non-polar for non-polar, etc.) that would be unlikely to affect opsin sensitivity regardless of their location (Nathans 1990; Nakayama and Khorana 1991). While further differences may have occurred outside of the region sequenced, i.e., in the N and C- terminal regions of the protein, studies indicate that variability in these portions of the opsin protein are unlikely to affect its spectral properties; thus, they were not evaluated for any of the genes examined (Hargrave 1982; Hunt et al. 2001; Shi and Yokoyama 2003).

The predicted spectral sensitivity for each rod opsin, as indicated by its wavelength of maximum sensitivity (λ_{\max}) was taken from the mutagenesis data of Fasick and Robinson (2000) for the sites 83, 292, and 299. These values are listed at the right side of Table 3. *M. angustirostris* was the only species examined to exhibit

Table 3. Summary of amino acid substitutions for obtained rod opsin sequences in comparison to the cat, dog, cow and a beaked whale. Putative tuning sites identified by Hunt et al. (2001) are shaded. Determination of synonymous (s) vs. non-synonymous (n) substitution type was made for marine carnivores in comparison to the cat and dog. λ_{max} values predicted in this study are shown in bold.

species	residue																				λ_{max}	measured	reference				
	36	46	53	53	104	107	158	159	162	163	182	217	239	286	290	292	297	298	299	315				319	332	335	
cat	y	L	V	D	V	P	A	F	V	L	M	I	I	I	L	A	S	S	S	S	N	T	A	T	501		
dog	y	L	V	D	V	P	A	F	V	L	M	I	I	I	L	A	S	S	S	S	N	T	A	A	501		
<i>Pugophilus groenlandicus</i>	y	L	V	D	V	P	A	G	L	V	L	M	I	I	L	A	A	A	A	A	T	T	V	A	499	497, 498	Lavigne and Ronald 1975, Fastick and Robinson 2000
<i>Phoca vitulina</i>	f	L	V	D	V	P	G	F	V	L	M	I	I	I	L	A	A	A	A	A	T	T	V	A	501	496, 501	
<i>Pusa hispida</i>	f	L	V	D	V	P	G	F	V	L	M	I	I	I	L	A	A	A	A	A	T	T	V	A	501		
<i>Erignathus barbatus</i>	f	M	I	D	V	P	A	F	V	L	M	A	I	I	L	A	G	A	A	S	T	T	V	A	501		
<i>Mirounga angustirostris</i>	f	L	I	D	V	P	A	L	F	L	L	T	I	I	I	S	S	A	A	A	N	I	-	-	487	486	Southall et al. 2000
<i>Leptonychotes weddellii</i>	f	L	V	D	V	I	P	A	L	V	L	L	T	V	T	A	S	A	A	A	N	T	V	A	499	498	Lythgoe and Dartnall 1970
<i>Hydrurga leptonyx</i>	f	L	V	D	V	I	P	A	F	V	L	L	T	V	T	A	S	A	A	A	N	T	V	A	499		
<i>Zalophus californianus</i>	f	L	V	D	V	V	A	F	V	M	I	I	I	I	L	A	S	S	S	S	N	T	A	V	499	497, 501	Cresticelli 1958
<i>Odobenus rosmarus</i>	s	L	V	D	V	V	A	A	F	V	L	M	I	I	I	A	S	S	S	S	N	T	A	V	499		
polar bear	s	L	V	D	V	V	A	A	F	V	L	M	I	I	I	A	S	S	S	S	N	T	A	A	501		
sea otter	y	L	V	D	V	P	A	F	V	L	M	I	I	I	L	A	S	S	S	S	N	T	A	A	501		
cow	a	L	V	D	V	P	A	F	V	L	M	I	I	I	L	A	T	S	A	N	T	A	T	499	499	Fastick and Robinson 2000	
beaked whale	y	L	V	D	V	V	P	A	F	V	L	M	V	V	L	S	S	S	A	N	T	V	T	485	484	Fastick and Robinson 2000	
synonymous / non-synonymous	n	s	s	n	n	s	s	n	s	s	s	n	s	n	s	n	n	n	n	n	s	n	n	n			

	10	20	30	40	50	60	70	80	90	
cat	MTQRWGPQRL	AGGQPHAGLE	DSTRASIFTY	TNSNATRGGP	EGPNYHIAPR	WVYVTSAWM	IFVVIASVPT	NGLVLAATMK	FKKLRHPLNW	ILVN
goat	.AHT Q.NF.	E. OG. S. D. L. V. R
P.gro	.A.T. D.R.QP.Y. Q. L. T. R
P.vit	.A.T. L. D.R.QP.Y. Q. L. T. V. R
P.his Q. L. T. V. R
E.bar Q. L. T. R
M.ang Q. A. L. I. R
H.lep Q. L. I. R
L.wed Q. F. I. R
Z.cal Q. L. I. R
O.ros Q. L. T. R
p.bear Q. L. T. R
otter Q. L. T. F. R
dolphin	.A.Q. F. QTSF. QG.V. S. D. D. L. V. V. L. I. P. M.
human-M	.A.Q. SL. RH.QDSY. QS. S. L. V.
human-L	.A.Q. SL. RH.QDSY. QS. S. L. V. T.
	110	120	130	140	150	160	170	180	190	
cat	AETIIASTIS	VVNQIYGYPV	LGHPMCVLEG	YTVSLCGITG	LWSLAIISWE	RMLVVCCKPFG	NURFDAKLAI	AGIAPSWIWA	AVWTAPPIFG	WSRY
goat M. L. V. M. T
P.gro I. L. V. M. V. V. S. L. R.
P.vit I. L. V. M. V. V. S. L.
P.his I. L. V. M. V. V. S. L.
E.bar I. H. L. V. M. V. V. S. L.
M.ang I. H. L. V. M. V. V. S. L.
H.lep I. H. L. V. M. V. V. S. L.
L.wed I. H. L. V. M. V. V. S. L.
Z.cal I. H. L. V. M. V. V. S. L.
O.ros I. H. L. V. M. V. V. S. L.
p.bear V. L. V. M. V. V.
otter V. L. V. M. V. V.
dolphin V. M. L. IV. F. M. S
human-M V. V. M. V.
human-L V. I. VS. V. V. S
	210	220	230	240	250	260	270	280	290	
cat	TSCGPDVFSG	SSYPGVQSYM	IVLMITCCII	PLSVIVLCYL	QVWLAIKAVA	KQKESSESTQ	KAEKEVTRMV	MVMIFAYCVC	WGPYTPFACF	AAAH
goat F. I. L.
P.gro T. G. I. V. V. F.
P.vit T. G. I. V. V. F.
P.his T. G. I. V. V. F.
E.bar T. G. I. V. V. F.
M.ang T. G. I. D. A. V. F.
H.lep T. G. I. V. V. F.
L.wed T. G. I. V. V. F.
Z.cal T. G. I. V. V. F.
O.ros T. G. I. V. V. F.
p.bear T. G. I. V. V. L. L.
otter T. G. I. V. L. L.
dolphin T. F. I. R V. L.
human-M V. T. I. V. L. F. F. A. N
human-L V. AI. M. V. L. F. F. A. N
	310	320	330	340	350	360				
cat	PLVAALPAYF	AKSATIYNPI	IYVFMNRQPR	NCIMQLFGKK	VDDGSELSSA	SRTEASSVSS	VSPA*			
goat L. S. V. K. *			
P.gro L. S. V. K. H. G			
P.vit L. S. V. K. *			
P.his L. S.			
E.bar L. S. K.			
M.ang L. S.			
H.lep L. S. K			
L.wed L. S. K.			
Z.cal L. S. K.			
O.ros L. S. K.			
p.bear L. S. K			
otter L. S.			
dolphin S. L. S. V. K.			
human-M M. P. V L. K. V. *			
human-L M. V. L. K. V. *			

Figure 8. Alignment of the obtained M/L cone opsin sequences, as well as those of the cat, goat, bottlenose dolphin, and human “green” (M) and “red” (L) cone opsins. Also included are the M/L cone opsins of *Pagophilus groenlandicus* and *Phoca vitulina* obtained from Genbank. Pinnipeds are listed by abbreviated forms of their binomial designation (see Table 2), other species by their common name. Dots (.) indicate identity with the feline sequence. Dashes (-) indicate gaps or missing data.

any substantial deviation in rod opsin sensitivity, with a λ_{\max} at least 12 nm shorter than that of any of the other pinnipeds. Two of the northern pinnipeds examined here had inferred λ_{\max} of 501, similar to that of a previously examined species, *Phoca vitulina* (Fasick and Robinson 2000), as well as the polar bear and sea otter. Except for *M. angustirostris*, the southern pinnipeds, as well as both the otariid and odobenid representatives had rod opsins with λ_{\max} of 499 nm. In all cases where sensitivity had previously been determined, inferences made solely from the variability at sites 83, 292, and 299 predicted rod opsin sensitivity to within 2 nm (Table 3).

Translated M/L cone opsin sequences are shown in Figure 8. In addition to the species examined in this study, M/L cone sequences for *Pagophilus groenlandicus* and *Phoca vitulina* from Genbank have been included for evaluation of sensitivity. A total of 27 sites varied in comparison to the sequences of two terrestrial mammals, the cat and the goat (*Capra hircus*). Two separate methods were used to infer M/L cone sensitivity from these variations in primary protein structure, the “five-sites rule” and a neural network appraisal.

First, Yokoyama and Radlwimmer’s (1998, 1999, 2001) five-sites rule was used to predict M/L cone sensitivity from five amino acid residues, 180, 197, 277, 285, and 308 (numbered from the human M/L cone opsin). According to this rule, the effects of substitutions at these sites are to shift spectral sensitivity in a predictable, independent, and additive fashion. Table 4 shows spectral sensitivity estimates according to the five sites rule as calculated from the human “red” M/L cone pigment with a λ_{\max} of 560 nm (Oprian et al. 1991). The effect of each substitution on the inferred λ_{\max} is shown. Both *Z. californianus* and *O. rosmarus* had residues identical to the human “red” at all five sites, and so the inferred λ_{\max} of their M/L cones is also 560 nm. All the phocid pinnipeds had one substitution from the human “red,” Y277F

(substitution of Y with F at residue 277), shifting the inferred M/L cone peak to 552 nm. The M/L cone of the polar bear is the same as those of the cat and goat at all five tuning sites, thus having an inferred λ_{\max} of 553 nm. Although the phocid pinnipeds had M/L cone peaks similar to those of terrestrial carnivores and polar bear, the combination of critical amino acids giving rise to their respective pigments were different at two of the five critical sites, A180S and Y277F. The sea otter M/L cone has slightly shorter peak sensitivity than that of the cat, with a λ_{\max} of 545 nm as a consequence of a single substitution, T285A. The M/L cones of *Z. californianus* and *O. rosmarus* are shifted even farther than those of the phocid pinnipeds and terrestrial carnivores because of the substitution A180S.

Robinson et al. (1999) have devised an alternative method to infer M/L cone sensitivity from sequence data using a neural network model. The model is restricted to M/L cone opsins possessing the same residues as either the human “red” or “green” M/L cone opsin at each of seven critical tuning sites, 116, 180, 230, 233, 277, 285, and 309. Note that for this method, human “red” and “green” cones were considered to have λ_{\max} of 561 nm and 533 nm, respectively (Asenjo et al. 1994). A summary of the residues possessed by each species at these seven sites as well as their inferred λ_{\max} is shown in Table 5. Sensitivity estimates for the M/L cones of *Z. californianus*, *O. rosmarus*, the polar bear and the sea otter were readily established according to this model, as these species possessed appropriate residues at all seven sites. The remaining six pinnipeds had appropriate amino acids at only five or six of the seven sites. Since glycine and serine are both hydrophilic polar amino acids of similar size, we made the assumption that G233 and S233 would be functionally similar and thereby estimated M/L cone sensitivity to be ~550 nm for *Pagophilus groenlandicus*,

Table 4. Sensitivity predictions for marine carnivore M/L cones according to the five-sites rule. Predicted λ_{\max} values are calculated from the human "red" M/L cone using the substitution values indicated. Values predicted in this study are shown in bold.

species	residue							λ_{\max}		reference
	180	197	277	285	308	predicted	observed			
cat	A (-7)	H	Y	T	A	553	553	Yokoyama and Radlwimmer 1999		
goat	A (-7)	H	Y	T	A	553	553	Yokoyama and Radlwimmer 1999		
<i>Pagophilus groenlandicus</i>	S	H	F (-8)	T	A	552	550	L. Newman, pers. comm.		
<i>Phoca vitulina</i>	S	H	F (-8)	T	A	552	550	L. Newman, pers. comm.		
<i>Pusa hispida</i>	S	H	F (-8)	T	A	552	-	this study		
<i>Erignathus barbatus</i>	S	H	F (-8)	T	A	552	-	this study		
<i>Mirounga angustirostris</i>	S	H	F (-8)	T	A	552	-	this study		
<i>Leptonychotes weddellii</i>	S	H	F (-8)	T	A	552	-	this study		
<i>Hydrurga leptonyx</i>	S	H	F (-8)	T	A	552	-	this study		
<i>Zalophus californianus</i>	S	H	Y	T	A	560	-	this study		
<i>Odobenus rosmarus</i>	S	H	Y	T	A	560	-	this study		
polar bear	A (-7)	H	Y	T	A	553	-	this study		
sea otter	S	H	Y	A (-15)	A	545	-	this study		
dolphin	A (-7)	H	Y	T	S (-27)	526	524	Fasick and Robinson 1998		
Human - "green"	A (-7)	H	F (-8)	A (-15)	A	530	530	Oprian et al. 1991		
Human - "red"	S	H	Y	T	A	560	560	Oprian et al. 1991		

Table 5. Sensitivity predictions for marine carnivore M/L cones according to the Robinson et al. (1999) neural network model. Red/green combinations are listed sequentially beginning with residue 116. For this, "r" and "g" indicate similarity with human "red" and "green" cones, respectively; uppercase letters identify the amino acid for non-matching residues. Human "red" and "green" cone values are from Asernjo et al. (1994). Predicted λ_{\max} values from this study are shown in bold.

species	residue										λ_{\max}	
	116	180	230	233	271	385	399	red/green combination	predicted	observed		
cat	Y	A	I	S	Y	T	Y	ggrrrr	554	553		
goat	Y	A	I	S	Y	T	Y	ggrrrr	554	553		
<i>Pagophilus groenlandicus</i>	Y	S	I	G	F	T	Y	grGrr	550	550		
<i>Phoca vitulina</i>	Y	S	I	G	F	T	Y	grGrr	550	550		
<i>Pusa hispida</i>	Y	S	I	G	F	T	Y	grGrr	550	-		
<i>Erignathus barbatus</i>	H	S	I	G	F	T	Y	HrrGrr	(550/554)	-		
<i>Mirounga angustirostris</i>	H	S	I	G	F	T	Y	HrrGrr	(550/554)	-		
<i>Leptonychotes weddellii</i>	H	S	I	G	F	T	Y	HrrGrr	(550/554)	-		
<i>Hydrurga leptonyx</i>	H	S	I	G	F	T	Y	HrrGrr	(550/554)	-		
<i>Zalophus californianus</i>	Y	S	I	S	Y	T	Y	grrrrr	558	-		
<i>Odobenus rosmarus</i>	Y	S	I	S	Y	T	Y	grrrrr	558	-		
polar bear	Y	A	I	S	Y	T	Y	ggrrrr	554	-		
sea otter	Y	S	I	S	Y	A	Y	grrrrr	543	-		
dolphin	Y	A	I	S	Y	T	Y	ggrrrr	554	524		
Human - "green"	Y	A	T	S	F	A	F	ggrrrrrr	533	533		
Human - "red"	S	S	I	A	Y	T	Y	rrrrrr	561	561		

Phoca vitulina, and *Pusa hispida*. For all the above species, predicted λ_{\max} was within 2 nm of estimates made with the five-sites rule. For four pinnipeds, it was essentially impossible to evaluate sensitivity with the neural network model. *E. barbatus*, *M. angustirostris*, *H. leptonyx* and *L. weddellii* all have histidine at residue 116. Histidine is a positively-charged, polar amino acid and thus dissimilar from the uncharged tyrosine or serine residues. For comparison, two estimates are given in Table 5 presuming the site similar to both Y116 and S116. However, although residue 116 does not occur in a transmembrane region and thus should have a relatively minor effect on spectral tuning (Asenjo et al. 1994), these values should be considered approximations only, as the effect of the substitution H116 has not been evaluated.

In addition to the sites involved in these models, 20 other variable sites were found among the obtained M/L cone sequences (see Figure 8). Of these, only four were substitutions that would affect the electrochemical nature of the residue. The other 16 substitutions were synonymous changes and thereby insignificant to the determination of spectral sensitivity. Of the four non-synonymous changes, one, A58T, occurred in the N-terminal region of the opsin and two were found only in the sea otter. Thus, none of these changes would likely affect pinniped M/L cones. The final change, P187R in *Pagophilus groenlandicus*, is also unlikely to have an effect, as this change does not occur in *Phoca vitulina* and these two species have the same M/L cone sensitivity, λ_{\max} 550 nm (L. Newman, pers. comm.).

As discussed, no S cone opsin sequences were amplified from the retinal mRNA/cDNA of any of the pinnipeds. However, S cone data was obtained for the sea otter and the polar bear. Table 6 summarizes the amino acid condition of both species for the nine sites that are thought to be critical to determining the spectral properties of S cone opsins (Fasick et al. 2002; Shi and Yokoyama 2003). Similar data are shown

for two other terrestrial mammals, cow and human. No data is currently available for any terrestrial carnivores. As shown, both the sea otter and polar bear have the same residues as the cow at seven of the nine critical sites. In addition, at one of the two variable sites, residue 118 (numbered according to the bovine S cone opsin), polar bear, sea otter, and cow all have hydrophilic polar residues, i.e., non-synonymous substitutions. Finally, at residue 93, both the sea otter and polar bear have a non-synonymous substitution previously shown to cause a 3 nm long-wavelength shift in sensitivity (Cowing et al. 2002). As this is the only apparent functional difference between the S cones of the two carnivores and the cow, the inferred λ_{\max} for both the sea otter and the polar bear is 441 nm.

Table 6. Summary of the condition of all putatively significant amino acid substitutions (Shi and Yokoyama 2003) of the S cone opsins of polar bear, sea otter, human, and cow. The spectral significance (shown in parens.) of the substitution at site 93 is in reference to the S cone of the cow.

species	residue									λ_{\max}	reference
	86	89	92	86	90	93	114	116	118		
cow	F	F	T	Y	S	I	A	L	C	438	Chiu et al. 1994
polar bear	F	F	T	Y	S	T (+3)	A	L	S	441	this study
sea otter	F	F	T	Y	S	S (+3)	A	L	T	441	this study
human S "blue"	T	L	F	L	S	P	G	L	T	414	Fasick et al. 1999

ERG MEASUREMENTS

In earlier ERG measurements made on a pair of *Phoca vitulina* evidence was found for a spectral mechanism having a λ_{\max} value of about 510 nm and an absorption spectrum with a shape appropriate for a single photopigment (Crognale et al. 1998). Since this mechanism was detected under test conditions that have typically yielded indications of photopic activity in many other types of mammal (e. g., light adapted eye, flickering stimulus lights), this was concluded to represent a contribution to the ERG from an M/L cone photopigment. The estimated peak of that mechanism, however, is very different from that predicted from the opsin sequence data (Tables 4, 5), the latter implying that the *P. vitulina* M/L pigment should have a spectral peak value of 550-552 nm. That striking disparity stimulated additional measurements on *P. vitulina* and on two other types of pinnipeds.

To attempt to provide stimulus conditions even more favorable for eliciting photopic signals, the ambient illumination in the laboratory was increased from that of the previous experiment (from 100 to 495 lux) and the intensity of the reference light was similarly increased by about a factor of four. With these changes in place spectral sensitivity of a *P. vitulina* was measured at two wavelengths, 500 nm and 550 nm. These measurements were made at four different flicker rates, ranging from 10 Hz to 25 Hz. Each pair of the sensitivity values obtained from this procedure was best fitted using standard photopigment absorption curves (Govardovskii et al. 2000) with the λ_{\max} values determined to the nearest 0.1 nm. The idea behind the experiment was that if the 510 nm peak value obtained earlier reflected mostly contributions from rods, then those signals should become progressively attenuated at higher flicker rates, i.e., one should see a Purkinje shift. Values obtained from six separate measurements on *P. vitulina* are shown in Figure 9. The straight line in that plot is the best-fit linear

regression. Note that the slope of this line does not differ significantly from zero. The only interpretation from this experiment is that over this range of pulse frequencies there is no significant shift in the spectral peak of the mechanism. The average peak value so measured across all stimulus frequencies was 502.5 nm. That value is very close to the peak predicted and observed *in vitro* for the rod pigment in this species (λ_{\max} 501 nm; Fasick and Robinson 2000) and somewhat shorter than that was measured in two *P. vitulina* previously tested, λ_{\max} 508 nm and 512 nm (Crognale et al. 1998). Finally, measurements were also attempted at frequencies beyond those represented in Figure 9, but it proved impossible to record any reliable ERG signals from the eye of *P. vitulina* at rates in excess of about 25 Hz.

Complete spectral sensitivity functions were measured using flicker-photometry for four *Zalophus californianus*. Figure 10 (top curve) shows the averaged results ($\pm 1SD$) obtained from three animals using 12.5 Hz flicker. The best-fitting photopigment absorption curve (continuous line) has a peak value of 501.3 nm. For all four animals complete spectral sensitivity curves at 12.5 Hz were also obtained with a considerably brighter reference light. Those data (Figure 10, middle curve) are well fit with a pigment having a peak value of 499.2 nm. The peaks of these curves are the same as that predicted for the *Z. californianus* rod pigment (λ_{\max} 499 nm; Table 3) and there is no hint that the brighter lights caused any shift toward the longer wavelengths as would be expected if there were both rod and cone contributions. In a further attempt to detect cone signals an experiment was conducted on *Z. californianus* that was similar to that described above for *P. vitulina*, i.e., by measuring spectral sensitivity across a range of stimulus frequencies. Sensitivity was measured at 500 and 560 nm at five stimulus frequencies ranging from 5 Hz to 30 Hz and the resulting data were best fit with photopigment absorption curves. The results are shown in the

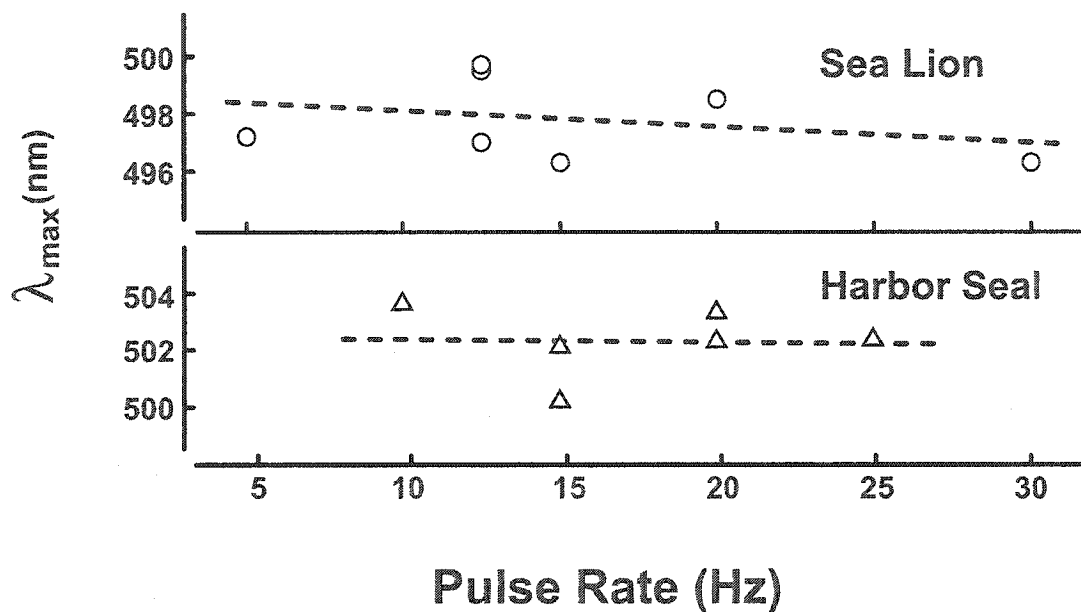


Figure 9. Spectral sensitivity as a function of flicker rate measured for two species of pinniped. Plotted for the harbor seal (*Phoca vitulina*) and the California sea lion (*Zalophus californianus*) are the peaks (λ_{\max}) of the photopigment absorption function obtained from best fits to a series of two-point spectral sensitivity functions (see text). The dashed lines are best-fit linear regressions; neither of these has a slope significantly different from zero.

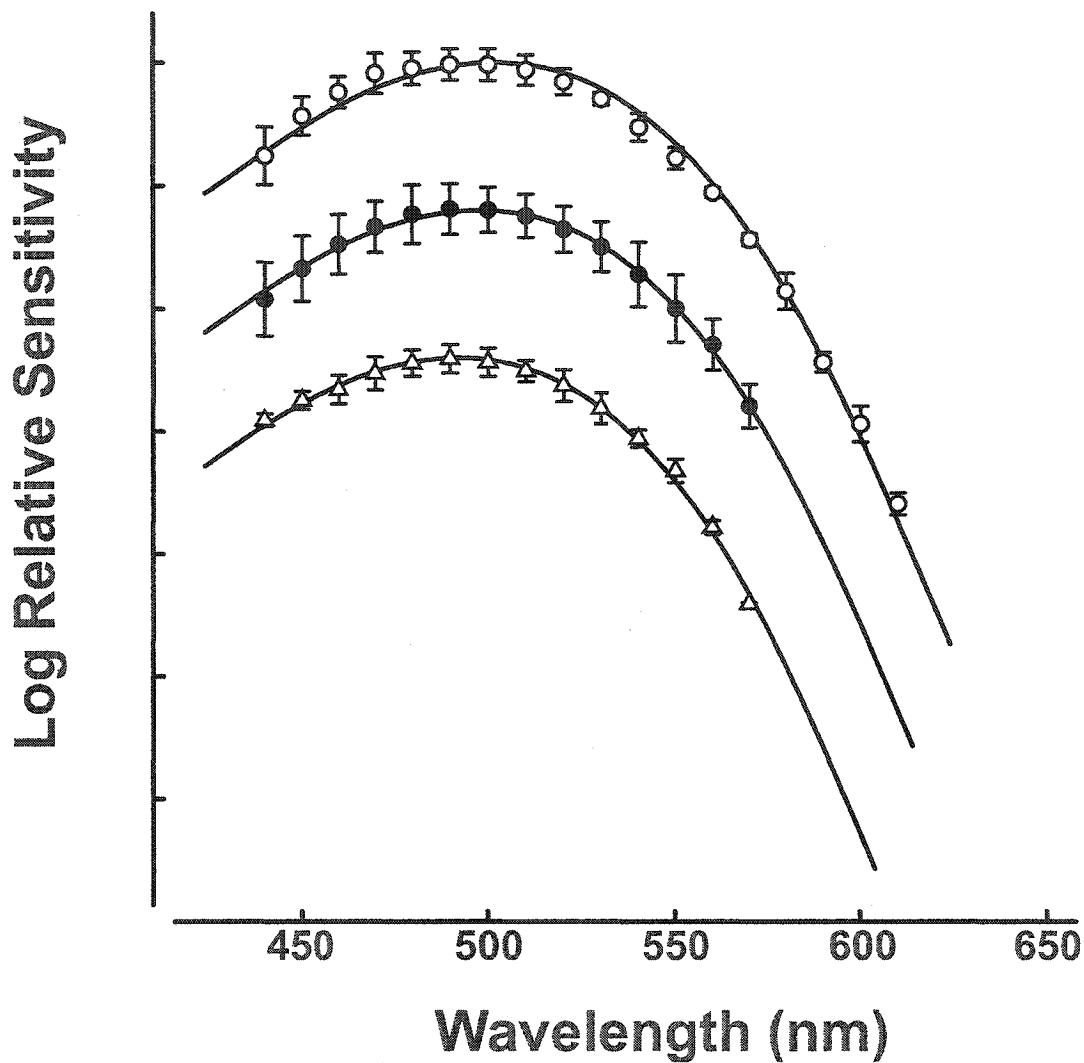


Figure 10. Spectral sensitivity functions for California sea lions (*Zalophus californianus*; open and filled circles) and northern elephant seals (*Mirounga angustirostris*; triangles) as measured with ERG flicker photometry. The data points are mean values for three animals (top), four animals with brighter reference light (middle) and two animals (bottom), respectively (see text). Error bars are ± 1 standard deviation. The lines are best-fitting photopigment absorption functions having peaks of 501.3 nm and 499.2 nm for *Z. californianus* and 491.7 nm for *M. angustirostris*.

top panel of Figure 9. As in the case of *P. vitulina* there is no hint of a shift in spectral sensitivity as a function of flicker frequency. Across seven separate tests, the average spectral peak was 497.8 nm with the individual values covering a total range of only 3.2 nm.

It proved a challenging task to gain good ERG recording from the two *Mirounga angustirostris* as the recorded signals were generally quite small. Nevertheless, it was eventually possible to make spectral sensitivity measurements for a single condition (12.5 Hz) for both animals. These data are shown in the bottom curve of Figure 10. Note that despite the difficulty in the recording the results from the two animals are virtually identical. The averaged values for the *M. angustirostris* tightly fit a photopigment absorption curve having a λ_{\max} of 491.7 nm. This short wavelength-shifted sensitivity is reasonably close to the inferred λ_{\max} of 487 nm and the observed λ_{\max} of 486 nm for this species (Southall et al. 2002). It was impossible to obtain any reliable ERG signals for flicker rates of 20 Hz and higher in this species.

Discussion

The high degree of nucleotide identity seen between the genes sequenced here and homologous rod, M/L, and S cone opsins indicate that these genes are members of the classes to which they have been ascribed. Their phylogenetic organization into appropriate groups further supports this hypothesis. In addition, the structure of the rod and M/L gene trees bear some significance upon hypothesized taxonomic relationships among pinnipeds and marine carnivores in general (Rice 1998; Berta and Summich 1999; Bininda-Emonds 2000). The monophyletic origin of the pinnipeds, once debated, is identically reflected in both the rod and M/L cone opsin gene trees (for a review see Bininda-Emonds 2000). Our data clearly indicate that the entire pinniped lineage descends from a single ancestor, an ancestor shared with the Ursidae.

Within the pinnipeds, the grouping of the Odobenidae with the Otariidae and this clade as sister taxa to the phocid pinnipeds are strongly supported by the bootstrap analysis and are similarly represented in both developed gene trees. The exact relations among the three pinniped families continues to be debated (e.g. Carr and Perry 1997; Rice 1998), as the walrus (Family Odobenidae) shares many morphological similarities with the phocids but genetic similarities with the otariids (Bininda-Emonds 2000). On two separate counts, our data support the genetic evidence that Otariidae and Odobenidae are sister groups. Within the phocidae, all species examined are appropriately grouped, with the “southern” monachine phocids distinct from the “northern” phocine sub-family, including the correct placement of the Northern elephant seal (*Mirounga angustirostris*) within the Monachinae (Rice 1998).

The presence of the obtained opsin genes within the retinal mRNA indicates that these visual pigment genes are being expressed in the retina. The absence of any introns or mis-sense mutations suggests that they are functionally utilized. Although behavioral observations are required to confirm this, there is no reason to suspect otherwise from the available data. Thus, we conclude that the presence of these mRNA sequences reflects the existence of functional rod and M/L cone photoreceptors. Similarly, our inability to amplify any S cone opsin sequences from the retinae of any of the pinnipeds suggests that these species are not producing S cone visual pigment, and thus they lack S cone photoreceptors. Of course, negative results such as these must always be interpreted cautiously. It is possible that there are so few S cones that amplification procedures were somehow inadequate for their detection. However, the relative ease with which these primers amplified sea otter and polar bear S cone sequences from mRNA and pinniped S cone sequences from nuclear DNA

suggests that the lack of S cone mRNA in the retina likely reflects its complete or near-complete absence rather than just our inability to amplify it. This conclusion is supported by previous reports of the absence of S cones in *Phoca vitulina*, *Pusa hispida*, and *E. barbatus* using other methods (Peichl et al. 2001, 2002).

The inclusion of an additional five pinnipeds to the list of species without S cones lends support to the hypothesis that the entire pinniped group lacks this cone type (Peichl et al. 2001). The absence of S cones has been documented in nearly half of all pinnipeds, now including numerous species from the phocid and otariid families and the only living member of the odobenid family. By comparison, among terrestrial mammals most species examined have been found to possess both S and M/L cone visual pigments (Jacobs 1993; Yokoyama and Yokoyama 1996). On land, the loss of S cones is relatively rare, and is limited to isolated occurrences at the most distal branches of the primate, carnivore, and rodent lineages (e.g., Jacobs et al. 1996b; Peichl and Moutairou 1998). In contrast, within pinnipeds the loss of S cones is widespread. So much so, in fact, that it seems probable that the loss occurred before the divergence of the three pinniped families. Indeed, a single mutational occurrence at a basal point in the pinniped lineage is probably the most parsimonious explanation for the widespread loss of S cones among pinnipeds. A similar phenomenon has recently been documented to underlie the loss of S cones among cetaceans (Levenson and Dizon 2003).

The convergent loss of S cones in pinnipeds and cetaceans suggests that in marine mammals the loss of this cone type is linked to adaptation for aquatic vision. Among terrestrial mammals, the few species known to lack S cones are strongly nocturnal and/or subterranean in habitat (Jacobs and Deegan 1992; Jacobs et al. 1996a). Pinnipeds are neither. Numerous hypotheses have been put forth to explain

the adaptive significance of the loss of S cones for aquatic vision, though none have been explicitly tested (e.g. Peichl et al. 2001; Cowing et al. 2002; Griebel and Peichl 2003). If an adaptive explanation is found, it is likely that it will involve the determination that S cone sensitivity is detrimental to aquatic vision. The loss of S cones would not only reduce sensitivity to short wavelength light, it would also eliminate the ability to make cone-photoreceptor based color discriminations. Given that, there must be a substantial disadvantage to S cone function to offset the loss of these other valuable visual abilities. Interestingly, the third major group of marine mammals, the herbivorous sirenians, have maintained functional S cones and the associated ability for color discrimination (Griebel and Schmid 1996; Peichl et al 2001). Many fish species have also retained S cones, though they are much less common in species from deeper waters (Bowmaker 1998). Perhaps the loss of S cones is not just an adaptation for aquatic existence; rather, it may be more specifically tied to behavior such as foraging modality or diving. The adaptive significance of the retention of S cones by the sea otter and polar bear is difficult to interpret in this scenario, as neither have had very long to evolve for life in the marine environment: the sea otter separated from its non-marine relatives just 3.5 MYA (Estes et al. 2002), the polar bear less than 1 MYA (Stirling 2002). Indeed, the S cones of both these species are essentially the same as those of other terrestrial carnivores (Jacobs et al. 1993).

Despite some behavioral and physiological evidence to the contrary (Lavigne and Ronald 1972; Crognale et al 1998) genetic evidence predicts that photopic vision in pinnipeds is based on a single M/L cone pigment with maximum sensitivity of 550-560 nm. These estimates have recently been confirmed for *Pagophilus groenlandicus* and *Phoca vitulina* using *in vitro* expression techniques (L. Newman, pers. comm.).

These M/L cone pigments are similar in sensitivity to those of terrestrial carnivores and quite different from the short-wavelength shifted M/L cones of the bottlenose dolphin (*Tursiops truncatus*; Table 4). Presuming the shifted sensitivity of the dolphin's M/L cones is a consequence of adaptation to the relatively short wavelengths of light present under water, then clearly the M/L pigments of pinnipeds have not undergone aquatic adaptation. In fact, the M/L cones of *Z. californianus* and *O. rosmarus* are slightly long-wavelength shifted in sensitivity in comparison to those of most other carnivores (Yokoyama and Radlwimmer 2001). In addition, parsimonious interpretation of the genetic data indicates that active selective processes have maintained the long-wavelength sensitivity of pinnipeds; i.e., it is not simply a consequence of phylogenetic inertia and maintenance of the ancestral terrestrial condition. Despite nearly identical inferred sensitivities, the M/L cones of the pinnipeds have different residues at several of the identified spectral tuning sites even in comparison to their very close relative, the polar bear. In the five-sites model, for instance, all seven phocid species have undergone a long-wavelength shift in sensitivity, A180S, as well as a short-wavelength shift, Y277F. Similarly, in the Robinson *et al.* (1999) model, despite nearly identical predicted sensitivities, the phocid pinnipeds possess three or more substitutions from the sequences of terrestrial carnivores.

The exclusive presence of relatively long-wavelength sensitive M/L cones in pinnipeds indicates that these animals have limited photopic sensitivity to short-wavelength light. In addition, as the ability to discriminate color is based on the presence at least two photopigment types, pinnipeds must be incapable of cone-based color vision. This conclusion is counter to behavioral reports that pinnipeds can make color discriminations (Wartzok and McCormick 1978; Busch and Ducker 1987;

Griebel and Schmid 1992). It is possible that these anomalous behavioral results are simply the consequence of complications associated with the difficulty of working with live, trained animals. It is perhaps noteworthy in this regard that there was considerable inter-animal variability in color discrimination capacity in some of these studies. Alternatively, pinnipeds may be capable of the simultaneous employment of rod and cone signals thus providing them with an atypical, albeit not unheard of (e.g., Alpern et al. 1971) form of dichromatic color vision (Crognale et al. 1998). Pinnipeds do possess remarkably contractile pupils capable of up to 250-fold changes in aperture size (Levenson and Schusterman 1997). This is perhaps sufficient to limit retinal illuminance levels in some photopic conditions so that rod photoreception can occur without total bleaching.

Like the M/L cone pigments, the rod visual pigments of pinnipeds bear little resemblance to those of cetaceans. The initial discovery of a “deep-sea” rhodopsin in *Mirounga leonina* (Lythgoe and Dartnall 1970) raised the possibility that pinnipeds might possess short-wavelength shifted rod pigments to increase sensitivity to the wavelengths of light found under water. The entire cetacean order has blue-shifted pigments with λ_{max} of 492 nm or lower (McFarland 1971; Fasick and Robinson 2000), as do a wide variety of fish species, particularly those from meso- and bathy-pelagic habitats (Hunt et al. 1996; Bowmaker 1995, 1998). Given that, it would seem reasonable that pinnipeds, especially those that dive deeply or spend extended periods of time under water might have similar shifts. However, with the addition of the data reported here, roughly half of the pinniped group has now been examined including most of the deeper-diving species, and it appears that pinniped rod visual pigments are actually quite similar to those of terrestrial mammals, with λ_{max} very near 500 nm.

The short-wavelength shifted rod pigments of *M. leonina* and *M. angustirostris* are the exception rather than the rule.

However, the similarities between terrestrial mammals and other pinnipeds in rod opsin sensitivity do not appear to be solely a consequence of phylogenetic inertia. There are 20 amino acid substitutions that have arisen in these rod opsin genes since pinnipeds diverged from their terrestrial ancestors; yet, except for sites 83, 292, and 299, all of these changes occurred at spectrally insignificant locations. Moreover, those mutations that did occur at spectrally significant sites had little effect on the sensitivity of the opsin. Many of the pinnipeds have different combinations of spectral tuning residues than their terrestrial relatives, yet these animals have maintained functional rod opsins with λ_{\max} near 500 nm. At the critical sites 83, 292, and 299, a substitution to DAA in *Pagophilus groenlandicus*, *L. weddellii*, and *Hydrurga leptonyx* shifts the λ_{\max} of their rod opsin -2 nm to 499 nm. Expression and analysis of this opsin *in vitro* confirms this inferred sensitivity (Fasick and Robinson 2000). The otariid and odobenid species have a different combination, NAS, but the same inferred λ_{\max} of 499 nm, previously confirmed by spectrophotometric evaluation of *Z. californianus* (Cresticelli 1958). In fact, only three pinniped species, *Phoca vitulina*, *Pusa hispida*, and *E. barbatus*, in addition to the polar bear and the sea otter, possess the same combination of critical amino acids as the terrestrial carnivores, though they all have essentially the same rod opsin sensitivity.

The similarity between both the M/L cones and rod opsins of pinnipeds and terrestrial mammals might be ascribed to several factors. Perhaps ambient conditions under water are sufficiently dim to limit the utility of vision while diving, such that the visual pigments of pinnipeds have not been under selective pressure to be modified for aquatic use. The short-wavelength shifted rods and rapid dark-adapting abilities of

elephant seals, and other dramatic sensitivity-increasing adaptations generally seen in pinnipeds suggest otherwise (Walls 1942; Lavigne et al. 1977; Levenson and Schusterman 1997, 1999), as do observations of visually oriented foraging by pinnipeds (Hobson 1966; Davis et al. 1999).

For some species, shifting rod pigment sensitivity to shorter wavelengths would provide little selective advantage. For *Phoca vitulina*, *Pusa hispida*, or *Z. californianus*, the shallow coastal waters they inhabit can have maximum light transmission at wavelengths near or above 500 nm (Kirk 1994). However, in addition to the shallow-water, coastal species, the pinnipeds studied here represent a variety of other habitats, including high latitude benthic (*O. rosmarus*), pack-ice (*H. leptonyx*, *Pagophilus groenlandicus*) and polar deep-water environments (*L. weddellii*), all of which have different spectral properties (Jerlov 1976; Kirk 1994). By comparison, all cetaceans, including even the shallowest-diving coastal species, possess rod pigments with λ_{\max} shorter than 493 nm (Fasick and Robinson 2000). Thus, it is more likely that the similarity in rod opsins is a consequence of the terrestrial habits of pinnipeds. Unlike cetaceans, pinnipeds still spend significant portions of their lives out of the water. They give birth on land or ice, and most species remain ashore for substantial periods of time to rest, breed, nurse their offspring, and molt. During this time they are involved in often complex intra-species interactions and may face the threat of terrestrial predators. The tele-receptive properties of the visual sense would clearly be quite valuable in these conditions. Indeed, the similarity of pigments among species from such a wide range of aquatic habitats argues this point: pinniped opsins appear primarily adapted to provide good terrestrial visual sensitivity.

ERG MEASUREMENTS

The peaks predicted for pinniped rod pigments based on amino acid sequence (Table 3) agree with inferences drawn from the ERG measurements. The average of the several estimates made for *Phoca vitulina* tested at different stimulus frequencies is 502.5 nm, which compares well with the prediction of 501 nm from the genetic data. The several different estimates made for *Z. californianus* from both full spectra and from two-point spectral sensitivity functions average to 499.4 nm, essentially identical to that inferred from the sequence information. Finally, the spectra measured for two *M. angustirostris* had an average peak of 491.7 nm which also compares well with the 487 nm peak predicted from the genetic data. The *in situ* measurements seem to yield slightly longer peaks than the genetic predictions, especially for the elephant seal. However, deviations of this sort are not unique to pinnipeds. For example, ERG measurements of rod signals in the dog (Jacobs et al. 1993) suggest a spectral position that is longer than that predicted by tuning models. Reconciliation of these differences may well be unique for each case, but it is worth remembering that *in situ* measurements like these ERG recordings are influenced not only by the pigment spectrum, but also by any intraocular filtering and reflectivity. In general all of these factors will tend to displace spectral sensitivity curves toward the longer wavelengths. That said, the agreement between the direct measurements and tuning model predictions are remarkably close for the pinniped rod pigments here examined.

Whereas there is good agreement between the predictions from rod opsin sequences and ERG spectral sensitivity for these pinnipeds, this was not the case for cones. Indeed, there was no clear evidence in the ERG signals of any contribution from the cone pigments predicted from the molecular genetic results. This is particularly surprising, because these very same ERG procedures have been successfully employed to access cone-generated signals in a very wide range of other

mammals (Jacobs 1993). It has been known for well more than half a century that rod and cone contributions to the ERG can be separated by using flickering lights with rod contributions being superceded by cone contributions as flicker rate is increased (Adrian 1945). The current clinical standard for measuring human cone-based vision with the ERG specifies 30 Hz flicker (Marmor and Zrenner 1999), but cone spectra emerge from ERG flicker measurements at much lower rates than that. For example, flicker rates as slow as 12.5 Hz yield clear cone-based spectral sensitivity functions in rodents like the mouse and rat (Jacobs et al. 1991; 2001) and carnivores such as the ferret (Calderone and Jacobs 2003). The two-point spectral sensitivity functions (Fig. 4) obtained from *Z. californianus* and *P. vitulina* are particularly compelling in this regard. In these there is no hint of an intrusion of any cone contribution up to rates as high as 30 Hz. The absence of any cone contribution can be made explicit by calculating what would happen to measured spectral sensitivity in *Z. californianus* if there were joint rod and cone contributions. Given the estimated rod and cone peaks in this species (499 nm and 560 nm), combining the two in a proportion of 5:1 would shift peak spectral sensitivity from the rod peak of 499 nm out to 510 nm; even with the rod:cone ratio set at 9:1 the sensitivity peak would shift to about 504 nm, a value fully 7 nm longer than was measured at a stimulus frequency of 30 Hz and 5 nm displaced from what was measured at any flicker rate. In short, no hint of cone signals was detected in the ERGs of these pinnipeds with test conditions that routinely elicit such signals in a range of terrestrial mammals.

Is there something unique to pinniped eyes that render particularly difficult the recording of cone signals with the ERG? For instance, one might assume that perhaps the cone population is simply too small to yield reliable signals. Although there is no information for either *Z. californianus* or *M. angustirostris*, opsin antibody labeling

has been used to assess photoreceptor numbers in numerous pinnipeds, including *P. vitulina* and several other phocid and otariid species (Peichl and Moutairou 1998; Peichl et al. 2001). In these studies, M/L cones reach peak density values of 7000 to 11,000/mm². These values are no different from cone densities seen in some terrestrial carnivores from which ERG cone signals have been recorded, e.g., dog and ferret (Jacobs et al. 1993; Calderone and Jacobs 2003). Thus, it is not obvious that a paucity of cones can account for the recording failure. Although there is no evidence for it, it is also possible that the physiological integrity of the outer retina in these diving mammals is particularly susceptible to the effects of anesthesia. Isoflurane and halothane are known to have detrimental effects upon the bipolar cells of the retina in some species (e.g., Wassershaff and Schmidt 1986; Tremblay et al. 2003). If that were the case then, somehow, these effects would have to be more pronounced for cone than for rod-based signals. Finally, although it may seem unlikely, I can't exclude the possibility that the failure to record cone-based signals in the ERG accurately reflects events in the retina and thus predicts that these receptors contribute little to the visual lives of pinnipeds. Again, good behavioral studies of vision in these amphibious mammals are needed to assess that possibility.

In view of the combined findings from genetics and ERG, it is my opinion that earlier reports of photopic sensitivity and cone-based color discrimination in pinnipeds were confounded by the inadvertent inclusion of rod photoreceptor responses, despite testing under apparently photopic conditions. The pinnipeds examined here uniformly lacked S cones, leaving them relatively insensitive to short-wavelength light, and subsequently without the ability to make cone-photoreceptor based color discriminations. The distribution of the S cone loss suggests that the entire pinniped group probably lacks this cone type, a loss that seems to be related to adaptation for

aquatic vision. In contrast, the rod and M/L cones of pinnipeds are very much like those of terrestrial carnivores, except for the relatively short-wavelength sensitive rod opsins of the elephant seals. This similarity with terrestrial species is most probably related to continued selective pressure to maintain good terrestrial vision, although potential benefits for aquatic vision may also be realized for some species. Overall, the visual pigment complements of pinnipeds are exactly as their amphibious nature would predict, they have rod and M/L cone sensitivities like those of terrestrial carnivores, but lack short-wavelength sensitive cones like the entirely aquatic cetaceans.

Acknowledgements

Principal thanks go to the marine mammal stranding program of SeaWorld, San Diego, CA. Their program provided rehabilitated animals for electroretinographic (ERG) examination, as well as tissue samples from several species for genetic study. Thanks to Nick Sossin, Roshy Tajrishi, and especially Christine Levenson for help caring for the seals and sea lions at SIO and through their release. Representatives of the U.S. Fish and Wildlife Service (Fairbanks, AK), Alaska Department of Fish and Game (Anchorage, AK), California Department of Fish and Game (Santa Cruz, CA), and the Siniff seal research group (McMurdo sound, Antarctica) also provided valuable samples for genetic examination. Thanks to Carrie LeDuc for help developing laboratory protocols and to Roshy Tajrishi for assistance with the collection of opsin sequence data. We appreciate the valuable input of those who reviewed this manuscript. Graduate student support for DHL provided by NSF Office of Polar Programs (NSF 9814794). Financial support for this project provided by the San Diego Achievement Rewards for College Scientists (ARCS) program to DHL, the UCSD Chancellors Fund to PJP, and an NIH grant (EY002052) to GHJ.

The text of this chapter will be submitted for publication. I was the primary researcher and author and the co-authors listed in this publication directed and supervised the research which forms the basis for this chapter.

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V. CHAPTER FOUR

Penguin and sea turtle rhodopsins: A genetic comparison

Abstract

To evaluate whether deep-diving birds and sea turtles have adapted their visual pigments from those of their shallower-diving counterparts, the rod visual pigments (rhodopsins) of three penguins and five sea turtle species were compared. Rhodopsin nucleotide sequences were obtained from retinal mRNA for each species using genetic techniques. Spectral sensitivity of each rhodopsin was then determined through a comparative evaluation of primary protein sequence. Both the king and emperor penguin had rhodopsins similar to that of the shallow-diving Humboldt penguin with inferred maximum sensitivity (λ_{\max}) of ~504-506 nm. Similarly, none of the deeper-diving sea turtle species, including the very deep-diving leatherback turtle, had rhodopsins that were functionally different from that of the shallow-diving green turtle. All five turtle species studied had inferred λ_{\max} of ~502 nm. The absence of any short-wavelength shift in sensitivity, a character typical of the rhodopsins of meso/bathypelagic taxa, suggests that either vision is of limited significance to these deep-diving birds and turtles at depth, or that other factors have limited their ability to become specialized for vision in the deep sea.

Introduction

Among living birds and reptiles, several different groups of animals have evolved for a marine existence. The sea turtles are perhaps the most ancient of these; they separated from their terrestrial/freshwater relatives more than 150 million years ago to re-invade the marine environment (Pritchard 1997). At present there are seven sea turtle species distributed circum-globally throughout the tropics, with one species ranging into more temperate latitudes. Sea turtle foraging habitats vary widely among species, although most rarely leave the euphotic region of the water column (upper ~50 m; see Lutcavage and Lutz 1997). A few species may dive to much greater depths: loggerhead and olive ridley turtles can dive to more than 200 m; leatherback sea turtles have been recorded at depths in excess of 1000 m and may stay under water for over 30 minutes (Eckert et al. 1989; Lutcavage and Lutz 1997).

Among birds, the only group that shows a comparable degree of adaptation to the marine environment is the penguins, family Spheniscidae. The penguins are a monophyletic group that diverged from other lariid birds, probably in the early tertiary about 60 million years ago (O'Hara 1989; Sibley et al. 1989). During their extensive adaptation for a marine existence, penguins have dramatically improved their underwater locomotory capabilities, although at the expense of a complete loss of the ability to fly. Like sea turtles, most penguin species are also relatively shallow divers. Humboldt penguins, for instance, feed at depths of less than 50 m, rarely diving for more than a few minutes at a time and always returning to land within a few days (Wilson et al. 1989). In contrast, emperor penguins have been found diving to depths in excess of 500 meters and can stay under water for more than 10 minutes on a single dive (Robertson et al. 1993; Kooyman and Kooyman 1995). In addition, emperor penguins can remain at sea for several weeks at a time, as can their slightly shallower-

diving, sub-Antarctic relatives, the king penguins (Kooyman et al. 2000; Handrich et al. 1997).

There is evidence that the eyes of both penguins and sea turtles are morphologically adapted for use under water (Walls 1942; Northmore and Granda 1970; Sivak and Millodot 1977), and vision is clearly employed there, at least by shallow-diving, diurnally active species (e.g., Ponganis et al. 2000). However, with increasing depth ambient illumination decreases rapidly and available light becomes nearly monochromatic, centered on a relatively short-wavelength region of the visible light spectrum, $\lambda \sim 470$ nm (Kirk 1994). Consequently, in these light-limited conditions deep-diving marine birds and turtles might benefit from scotopic (dim-light or rod photoreceptor) visual pigments (rhodopsins) adapted to better match the spectrum of available light. It is for this reason that the maximum spectral sensitivity (λ_{\max}) of the rhodopsins of many deep-sea fish and deep-diving marine mammals are modified to have λ_{\max} closer to 470 nm. By comparison, similar species from shallower depths typically have λ_{\max} at or just above 500 nm (Bowmaker 1995; Fasick and Robinson 2000; Southall et al. 2002).

In both penguins and sea turtles, the visual pigments are composed of a light sensitive chromophore, 11-*cis* retinal bonded to a protein moiety, opsin (Wald 1968; Granda and Dvorak 1977; Bowmaker and Martin 1985). Variation at select locations on the opsin protein can affect the electrostatic interaction between the protein and the chromophore, which can in turn alter the absorptive properties of the pigment (see Fasick and Robinson 2000; Yokoyama 2000). For rhodopsin, all residues with the potential to alter the spectral properties of the visual pigment have been identified (Hunt et al. 2001). In addition, the precise effects of substitutions at many of these spectrally significant sites have been quantified (e.g. Yokoyama and Radlwimmer

1998; Fasick and Robinson 2000). To assess whether there are systematic differences between the rhodopsins of deep- and shallow-diving penguin and sea turtle species, we have used molecular genetic techniques to evaluate the rhodopsins of three penguin and five sea turtle species. The deepest-diving members of each group have been examined, as well as one or more shallower-diving species from each.

Methods

Penguin and sea turtle rod opsin (rhodopsin) nucleotide sequence data was obtained from retinal messenger RNA (mRNA). All eyes used for molecular analysis were collected from animals that died of natural causes. Rhodopsin sequences were obtained for three penguin species: emperor penguin (*Aptenodytes forsteri*; n=3), king penguin (*Aptenodytes patagonicus*; n=1), and Humboldt penguin (*Spheniscus humboldti*; n=4). Five sea turtle species were also examined: green turtle (*Chelonia mydas*; n=3), leatherback turtle (*Dermochelys coriacea*; n=3), loggerhead turtle (*Caretta caretta*; n=3), hawksbill turtle (*Eretmochelys imbricata*; n=3), and olive ridley turtle (*Lepidochelys olivacea*; n=3).

Detailed descriptions of the extraction, gene amplification, and sequencing techniques can be found in Chapter 3. Briefly, retinæ were excised and homogenized in Trizol to extract the retinal mRNA. The mRNA was reverse transcribed to cDNA. After transcription, rhodopsin gene sequences were subsequently amplified and sequenced from the cDNA using polymerase chain-reaction (PCR) and dideoxy-terminator cycle sequencing. Oligonucleotide primers for PCR and sequencing were designed from homologous gene sequences of alligator (Smith et al. 1995), duck, and budgerigar (Heath et al. 1997). For each rhodopsin, two overlapping sequence fragments were amplified to provide sequence data for all but the most distal portions of the gene, including all currently described spectral tuning sites (Hunt et al. 2001).

Putative rod opsin sequences were aligned with homologues from other vertebrates and evaluated phylogenetically to confirm their identity. Nucleotide and amino acid sequence alignments were performed with ClustalW (Thompson et al. 1994) and confirmed by eye. Phylogenetic trees were determined using parsimony with bootstrapping on PAUP software (Swofford 2001).

Rhodopsin spectral sensitivity was inferred from the translated primary protein structure of each rod opsin protein. For the evaluation of sensitivity, the turtle and penguin sequences were compared to the rhodopsin of a member of their respective group with known spectral sensitivity. Green turtle rod opsin sensitivity, λ_{\max} 502 nm, was taken from Liebman and Granda (1971), as measured *in vitro* with microspectrophotometry (MSP). Humboldt penguin rhodopsin sensitivity, λ_{\max} 504 nm, was taken from the MSP measurements of Bowmaker and Martin (1985). All amino acid variations were recorded and then evaluated for potential effects upon the spectral sensitivity of the rod opsin visual pigment using previously collected mutagenesis and comparative data (e.g., Fasick et al. 2000; Hunt et al. 2001). Additional details of this procedure are given below as they apply.

Results

Putative rhodopsin nucleotide sequences were obtained for all species examined. A phylogenetic comparison of these sequences as well as those of the cow (accession number AH001149) and alligator (U23802) are shown in Figure 11. Also shown for comparative purposes are the RH2 cone opsin genes of salmon (AY214157), zebra fish (AB087805), pigeon (AH007731), and green anole (AH007735). The rhodopsin sequences obtained here clearly formed a distinct branching separate from the RH2 cone opsin genes. Within the rhodopsins, the bird and reptile rods are clearly organized into a single monophyletic group with the cow

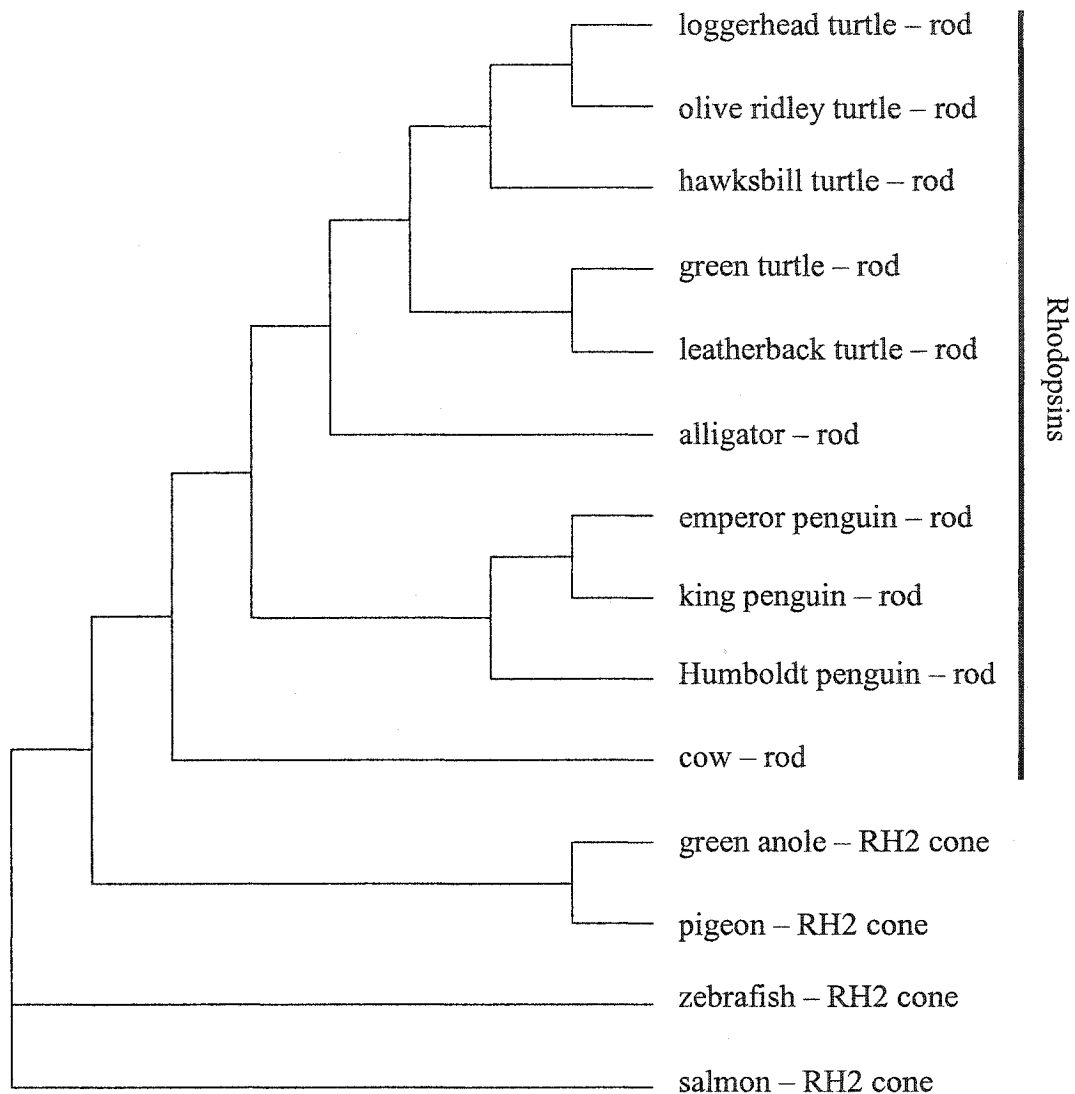


Figure 11. A phylogenetic tree created using parsimony with bootstrapping (1000 replicates). The rod opsins of all eight species examined here are included, as well as those of the alligator and cow from Genbank. Also, four RH2 cone opsins are shown for comparison: salmon (*Salmo salmo*), zebrafish (*Danio rerio*), pigeon (*Columbia livia*), and green anole (*Anolis anole*). Bootstrap values greater than 50% were retained. The rhodopsin portion of the tree is indicated at the right.

as outgroup. Within this, the three avian (penguin) species form a separate lineage from the alligator and sea turtles. Among the penguins, the congener emperor and king penguin form a sub-group that is sister to the Humboldt penguin. Within the turtles, the leatherback and green turtles are grouped separately from the other three species. These three are further subdivided by the exclusion of the hawksbill turtle.

A summary of all the amino acids that varied between the emperor and king penguin in comparison to the Humboldt penguin is presented in Table 7. Of the 13 sites that varied between these three species, five were non-synonymous substitutions. Non-synonymous substitutions are substitutions that affect the charge and/or polarity of the residue. It is this type of change that has been shown to affect the spectral sensitivity of the opsin protein, provided it occurs at a location where it may interact with the electrochemical environment of the opsin-chromophore bond (see Hunt et al. 2001). One of the five non-synonymous changes, A124G (substitution of A for G at residue 124) did occur at a spectrally significant location. This substitution has been shown to result in a relatively minor, ~2 nm long-wavelength shift in sensitivity (Hunt et al. 2001). As this was the only spectrally significant difference between the three species, the inferred λ_{\max} for the emperor and king penguin rhodopsins are thus ~506 nm, slightly longer than the 504 nm λ_{\max} of the rhodopsin of the Humboldt penguin.

Table 8 summarizes the amino acid variations observed in the rhodopsins of four sea turtle species when compared to the green turtle. Among the turtles there were 10 variable sites. Of these, four sites had substitutions that were non-synonymous. However, only one of the ten substitutions occurred at a spectrally significant site, I189V. As this is a synonymous change, it is not expected to affect the spectral sensitivity of the corresponding rhodopsin. Thus, all four other sea turtles

Table 7. Summary of all amino acids that varied between the Humboldt, king, and emperor penguin rod opsins. Non-synonymous changes are indicated. Spectrally significant substitutions are shaded.

species	83	88	116	123	124	137	165	169	194	198	217	263	281
Humboldt penguin	E	F	F	I	A	V	L	A	L	V	M	I	S
king penguin	D	L	C	V	G	I	F	T	P	I	T	I	A
emperor penguin	D	L	C	V	G	I	F	T	P	I	T	M	A
synonymous/non-synon	S	S	n	S	A	S	S	n	S	S	n	S	n

Table 8. Summary of all amino acids that varied between the rod opsins of five sea turtle species and the alligator. Non-synonymous changes are indicated. Spectrally significant substitutions are shaded.

species	92	103	107	134	168	173	189	274	281
green turtle	S	T	F	F	A	L	I	Y	S
leatherback turtle	S	T	F	F	A	L	I	Y	F
loggerhead turtle	N	T	F	F	A	F	V	H	S
hawksbill turtle	S	S	V	L	S	F	V	H	S
olive ridley turtle	S	T	F	L	A	F	V	H	S
alligator	S	T	F	I	A	V	V	Y	S
synonymous/non-syn	S	S	S	S	n	S	S	n	n

appear to have rhodopsins with the same λ_{\max} as the rhodopsin of the green turtle, 502 nm.

Discussion

In the obtained phylogenetic tree (Figure 11), the rhodopsin group is depicted as having been derived from the RH2 opsins, a type of cone visual pigment. This relationship has previously been demonstrated for other rhodopsins (Yokoyama 2000). Its replication here supports the conclusion that the genes examined in this study are in fact rhodopsins. The structure of the rhodopsin portion of the gene tree additionally supports conclusions about the relationships between the species examined in the study. The grouping of the emperor and king penguins, *Aptenodytes spp.*, as a sister clade to the Humboldt penguin concurs with previous phylogenetic evaluation of this family (O'Hara et al. 1989). For the turtles, previous studies have also found that the leatherback and green turtle are separate from the other species examined here (Dutton et al. 1996; Bowen and Karl 1997). The unusual skeletal and external morphology of the leatherback clearly supports this hypothesis (Bowen and Karl 1997). The inclusion of the green turtle with the leatherback as a separate subgroup is novel, though it may not necessarily be significant. Rather, it is probable that these two species have simply been clumped together here as a consequence of the relatively limited genetic information present in the highly conserved rhodopsin gene data set. The phylogenetic relationships of the three remaining species are as reported in previous genetic studies of sea turtles with the hawksbill turtle as outgroup to the loggerhead/olive ridley group (Dutton et al. 1996; Bowen and Karl 1997).

For the shallower-diving species examined in this study, the absence of any short-wavelength shifts in rhodopsin sensitivity is not surprising. The Humboldt penguin, green turtle, and hawksbill turtle are routinely shallow divers (<50 m), and

thus rarely experience the low-light and spectrally limited conditions that occur at greater depths (Wilson et al. 1989; Lutcavage and Lutz 1997). Moreover, many of these species are also generally diurnal in habitat and (particularly for the turtles) forage in relatively well-lit and often transparent waters. For these species, there is little need for shifting spectral sensitivity towards the shorter wavelengths of light that predominate at great depths. These species will rarely, if ever, experience these deep-sea conditions. The only scotopic conditions in which they will be active are those that occur at night in shallow water, where longer-wavelength light is still in relative abundance (e.g., Lythgoe 1979).

It is remarkable, however, that these data indicate little or no spectrally significant variation occurs among the deeper-diving species examined, as well. Diving/foraging strategies vary a great deal within these bird and turtle groups, including some species that regularly make very deep dives. In most other marine taxa that have been studied, there is a relatively clear correlation between rhodopsin spectral sensitivity and depth of habitat. This is particularly true for deep-dwelling species, such as the fish present at the great depths visited by the leatherback turtle and emperor penguin while deep diving (Bowmaker 1995; Douglass and Partridge 1997). Similar relationships between diving behavior and spectral sensitivity of rhodopsin have also been found in other diving tetrapods. For instance, in marine mammals, the deepest-diving pinnipeds show dramatic shifts in scotopic sensitivity (Southall et al. 2002), and in cetaceans there is a nearly linear correlation between rhodopsin λ_{max} and dive depth, despite considerable variability in other aspects of habitat and behavior (McFarland 1970; Fasick and Robinson 2000; Southall et al. 2002). For deep-diving emperor and king penguins and leatherback turtles, light levels will be greatly reduced at depth. If vision were of consequence to these species while diving, an appropriate

shift in rhodopsin spectral sensitivity would certainly be effective way to increase light sensing capabilities while diving there. Indeed, in addition to sunlight, much bioluminescent activity occurs at wavelengths around 475 nm (e.g., Widder et al. 1983), providing further benefit to species with maximum spectral sensitivity in this region of the spectrum.

The absence of short-wavelength shifted rhodopsins in these deep-diving birds and turtles raises an important question: why do they not exhibit adaptations for vision in the deep sea? Several possibilities seem worthy of consideration. Perhaps most obvious is the potential influence of phylogenetic inertia. Quite simply, it is possible that solely by chance, no adaptation has occurred at any of the spectrally significant sites. In other words, irrespective of their potential benefit, the mutational changes that would modify spectral sensitivity for deep-sea use may just have not occurred. Alternatively, the absence of adaptation might be a consequence of a more complex sort of phylogenetic inertia. The visual systems of sea turtles and penguins are principally photopically suited by design. They have relatively small lenses with correspondingly limited light-gathering abilities, and cone-dominated retinas (Northmore and Granda 1991; Bartol and Musick 2001). In fact, both penguins and sea turtles have light-filtering oil droplets in their retinas that additionally reduce sensitivity (Granda and Haden 1970; Liebman and Granda 1971; Bowmaker and Martin 1985). By comparison, marine mammals, like many terrestrial mammals (e.g., cat, dog, cow), have large, light-gathering lenses and rod dominated retinas (Walls 1942; Lavigne et al. 1977); they are much more scotopically adapted, to some extent as a consequence of ancestry. Deep-diving reptiles and birds are limited in this way. Although adaptation of their rhodopsins would certainly improve their visual

sensitivity deep under water, a number of other concomitant changes would also be required for them to be visually effective there.

Of course, while increased sensitivity would undoubtedly be beneficial, there is no reason to presume that the absence of adaptation for increased sensitivity is indicative of a complete inability to utilize vision at depth. There are a number of very deep-diving pinnipeds, for instance, that do not possess deep-sea visual pigments (Lavigne and Ronald 1975; Chapter 3). Recent observations have shown that one of these seals, the Weddell seal (*Leptonychotes weddellii*) is at least partially visually oriented even while foraging at great depths (Davis et al. 1999). Similarly, emperor penguins have been observed foraging visually below the sea ice of McMurdo Sound, although in this case not at great depths (Ponganis et al. 2000).

For the turtles, it is also possible that vision may not be of great consequence while diving deeply. In the case of the leatherback sea turtle, although many dives are made to extreme depths, they are much less common than some of the shallower dive types that might more reasonably be associated with foraging (Eckert et al. 1989; Lutcavage and Lutz 1997). These deeper dives may be resting dives or associated with the avoidance of visually oriented predators at shallower depths. Indeed, deep dives may be made to depths in excess of 1000 m, where sunlight-based vision is essentially impossible in even the clearest ocean waters. In either case, the turtles would not be searching for prey and might not need to be as reliant on vision. Other senses such as tactition and chemoreception might sufficiently replace vision in these scenarios. Alternate senses might also be important to king and emperor penguins at depth, although deep-diving behavior for these species is much more regular and is clearly associated with foraging (see Williams 1995).

Perhaps the most persuasive argument underlying the absence of adaptive evolution of scotopic sensitivity for marine use in sea turtles and penguins is the potential utility of rod-based vision on land. A short-wavelength shift in the spectral sensitivity of rhodopsin would be detrimental to terrestrial scotopic vision, where relatively long-wavelength light predominates. All three penguin species examined here are active on land, where they breed, lay eggs, and nurture chicks. During this time they must be alert, both day and night, for terrestrial predators, and they are constantly involved in sometimes complex intra-species interactions in the often extremely crowded and chaotic rookeries. Indeed, the need for scotopic vision on land is perhaps most extreme for the emperor penguins, where much of the breeding cycle takes place in the near complete darkness of the polar winter. For turtles, considerably less time is spent on land, although these terrestrial periods are similarly critical to their survival. Female turtles come ashore at night to construct nests and lay their eggs. Upon emerging from the nest, the hatchling turtles are faced with a brief, but critical visual task of safely returning to the ocean, typically at night-time. Experiments have shown that the visual sense is of considerable importance to both the nesting females and hatchling turtles in the completion of these tasks (Ehrenfeld and Carr 1967). Thus, it is possible that for penguins and/or turtles, an alteration of scotopic spectral sensitivity might be detrimental to the completion of the terrestrial aspects of their life history.

In summary, the genetic evaluation of rhodopsins from three penguins and five sea turtle species indicate that the rhodopsins of these species have spectral sensitivity with λ_{max} above 500 nm. There was no systematic variation in sensitivity between species with different diving behaviors. In fact, no short-wavelength shifting was observed in even the deepest-diving members of each group, despite the presence of

numerous amino acid substitutions between the different species. This absence of adaptation may be related to phylogenetic inertia, a link to terrestrial habitats, or perhaps to the reduced utility of vision in dim, underwater environments. While further study is needed to evaluate these potential underlying reasons, it is clear that the visual pigments of these sea turtles and penguins are not specialized for use while deep-diving.

Acknowledgments

Thanks to SeaWorld, San Diego, Southwest Fisheries Center, and P.J. Ponganis for assistance in obtaining appropriate tissue samples for examination in this study. Aimee Lang and Roshy Tajrishi were instrumental in the collection of data for this work. Some oligonucleotide primers used for amplification of penguin rhodopsins were provided by Dr. Karen Marchetti.

The text of this chapter will be submitted for publication. I was the sole researcher and author.

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