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Estimated absorbance spectra of the visual pigments of the North Atlantic right whale (*Eubalaena glacialis*)

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ABSTRACT

To assess the spectral sensitivities of the retinal visual pigments from the North Atlantic right whale (*Eubalaena glacialis*), we have cloned and sequenced two exons from the rod opsin gene and two exons from the middle-wavelength sensitive (MWS) cone opsin gene in order to determine the amino acids at positions known to be key regulators of the spectral location of the absorbance maximum (λ_{max}). Based on previous mutagenesis models we estimate that the right whale possesses a rod visual pigment with a λ_{max} of 499 nm and a MWS cone visual pigment with a λ_{max} of 524 nm. Although the MWS cone visual pigment from the right whale is blue-shifted in its spectral sensitivity like those from odontocetes, the spectral sensitivity of the right whale rod visual pigment is similar to those from terrestrial mammals.

Key words: *Eubalaena glacialis*, right whale, vision, retina, visual pigment, absorbance maxima, spectral sensitivity.

The underwater environment places unique constraints on the vision of cetaceans compared to their terrestrial mammalian counterparts. The visual sensory systems of cetaceans show numerous adaptations to an underwater light environment, limited in both photons and color, in order to increase photon capture. These adaptations include a spherical lens of great refractive power, an extensive tapetum lucidum (Prince 1956, Dawson 1980, Mass and Supin 2007) as well as a relatively large

cornea (Zhu *et al.* 2001) all employed to extend visual capabilities under low light conditions. These adaptations extend to the molecular range when the photoreceptor visual pigments are examined.

Visual pigments are light-sensitive molecules found in both the rod and cone photoreceptor cells of the vertebrate retina. Rods are typically associated with dim-light or night time (scotopic) vision while cones are associated with bright-light or day time (photopic) vision as well as color vision. Visual pigments consist of an integral membrane protein, opsin, combined with a chromophore that is covalently attached to the opsin protein at a lysine residue at position 296. Although the chromophore, 11-*cis* retinaldehyde in mammals, absorbs light maximally at 440 nm free in solution, the spectral location of this absorbance maximum (λ_{max}) is influenced by the amino acids of the surrounding opsin protein, resulting in visual pigments with a wide range of spectral sensitivities. For example, the human retina possess four classes of photoreceptor cells, each possessing a single visual pigment class, tuned to different regions of the visible spectrum (rod pigment or rhodopsin, 500 nm; blue or short-wavelength sensitive [SWS] cone, 414 nm; green or middle-wavelength sensitive [MWS] cone, 530 nm; and red or long-wavelength sensitive [LWS] cone, 560 nm) providing humans with monochromatic scotopic vision as well as trichromatic photopic color vision. Thus, it is the opsin protein and the different amino acid substitutions found in each opsin class that ultimately determine the absorbance spectra and resulting λ_{max} in each visual pigment class and the absorbance spectrum of the photoreceptor cell as a whole.

Cetacean visual pigments were first examined in detail by McFarland (1971), who determined that the rod visual pigments from many odontocete species were significantly blue-shifted in their spectral sensitivity relative to the rod visual pigments from terrestrial mammals. While terrestrial relatives of the cetaceans possess visual pigment λ_{max} values similar to those of the domestic cow (*Bos taurus*; rod λ_{max} = 500 nm, SWS cone λ_{max} = 438 nm, LWS cone λ_{max} = 552 nm; Yokoyama 2008), odontocetes possess blue-shifted rod visual pigments ranging in λ_{max} values from 484 to 488 as well as MWS cone visual pigments ranging in λ_{max} from 522 to 531 nm (Fasick *et al.* 1998, Newman and Robinson 2005). Unlike their terrestrial relatives, cetaceans lack a SWS cone class, resulting in the inability to distinguish spectral hues under photopic light conditions (Fasick *et al*. 1998, Peichl *et al.* 2001, Levenson and Dizon 2003). We can conclude from these observations that, like the visual pigments of deep-dwelling fish, deep-diving cetaceans have absorbance maxima matched to the wavelength of maximum spectral transmission of oceanic waters (Lythgoe and Dartnall 1970, McFarland 1971, Partridge *et al.* 1989) and that the cetacean retina is designed for photon capture, while the ability to discriminate spectral qualities or colors appears to be of no consequence.

Little is known about the rod and cone visual pigments from the mysticete whales. An early study determined that the rod visual pigment from the gray whale (*Eschrichtius robustus*) has a λ_{max} of 497 nm while that from the humpback whale (*Megaptera novaeangliae*) has a λ_{max} of 492 nm (McFarland 1971). The absorbance spectrum of the rod visual pigment from *E. robustus* is slightly blue-shifted when compared to those from terrestrial mammals and correlates well with the near-coastal photic environment in which this species lives (McFarland 1971). The absorbance spectrum of the rod visual pigment from *M. novaeangliae*, however, is intermediate to the rod visual pigments of *E. robustus* and the odontocetes. An examination of the dive profiles from two foraging *M. novaeangliae* individuals showed

dive depths with lunge feeding to occur between 40 and 100 m (Goldbogen *et al.* 2008), although *M. novaeangliae* has been known to dive to depths of 1,000 m (Norris 1969). Although *E. robustus* and *M. novaeangliae* possess rod visual pigments with different λ_{max} values, the visual pigments from these two species are well adapted to the available light found at their respective foraging depths. With the exception of the examination of the *E. robustus* and *M. novaeangliae* rod visual pigment absorbance spectra by McFarland (1971), no other mysticete rod or any mysticete M/LWS cone visual pigments have been investigated to date.

In order to understand the molecular mechanisms underlying the differences in absorbance spectra of the cetacean rod and cone visual pigments relative to their terrestrial relatives, mutagenesis models of both the bovine and dolphin opsin genes have been developed to determine which amino acids are responsible for differences in λ_{max}. Previous work has shown that a total of three amino acid substitutions fully explain the absorbance difference between the bovine ($\lambda_{max} = 500$ nm) and bottlenose dolphin (*Tursiops truncatus*, $\lambda_{max} = 488$ nm) rod visual pigments (Fasick and Robinson 1998). These substitutions are Asp83Asn, Ala292Ser, and Ala299Ser (bovine amino acid: position: dolphin amino acid). Combinations of the three dolphin amino acid substitutions have been incorporated into the bovine rod visual pigment in order to explain the range of λ_{max} values associated with other cetacean rod visual pigments including Sowerby's beaked whale (*Mesoplodon bidens*, $\lambda_{max} = 484$ nm) and *M. novaeangliae* ($\lambda_{max} = 492$ nm) (Fasick and Robinson 2000). Interestingly, the spectral placement of the cetacean MWS cone visual pigment is achieved by a somewhat different mechanism than that of the rod visual pigment. The dramatic blue-shift in λ_{max} associated with the *T. truncatus* MWS cone visual pigment ($\lambda_{max} = 524$ nm) when compared to the bovine LWS cone visual pigment ($\lambda_{max} = 552$ nm) is achieved by the single amino acid substitution Ala308Ser (Fasick and Robinson 1998). Although the contemporary mammalian M/LWS cone pigments are derived from the ancestral vertebrate LWS pigment ($\lambda_{max} = 560$ nm) by substitutions at three critical amino acid positions (180, 277, and 285; Yokoyama 2008, Yokoyama *et al.* 2008), the amino acids at these positions are identical between bovine and dolphin, allowing for only the Ala308Ser substitution to explain the 28 nm blue-shift.

In this study we present a strategy to estimate the spectral sensitivities of the *Eubalaena glacialis* rod and cone visual pigments by determining the amino acids at defined positions that influence the modulation of the absorbance spectra of both the rod and M/LWS cone visual pigments. Because of the lack of available fresh tissue from which mRNA could be extracted for *in vitro* expression and direct spectrophotometric measurement, we have instead obtained available genomic DNA samples in order to estimate the spectral sensitivities of the *E. glacialis* rod and cone visual pigments based on inferred amino acid sequences. We have cloned and sequenced exons 1 and 4 from the *E. glacialis* rod opsin gene to determine the amino acid substitutions occurring at positions 83, 292, and 299 as well as exons 3 and 5 from the *E. glacialis* MWS cone opsin gene to determine the amino acid substitutions occurring at positions 180, 277, 285, and 308. After incorporating the *E. glacialis* amino acids found at these positions into the mutagenesis models developed using the *B. taurus* rod and the *T. truncatus* MWS cone visual pigments (Fasick and Robinson 1998, Fasick and Robinson 2000), we estimate that *E. glacialis* possesses a rod visual pigment with a λ_{max} of 499 nm and a MWS cone visual pigment with a λ_{max} of 524 nm.

Materials and Methods

Identification of Deduced Amino Acid Substitutions from E. glacialis *Genomic DNA*

Genomic DNA from two North Atlantic individuals (Lab ID/Field ID: 13086/MH99-601-EG/Wellfleet, MA, U.S.A and Z28311/CCS-01E02/MA, U.S.A. [Marine Mammal and Sea Turtle Genetic Archive, National Marine Fisheries Service, Southwest Fisheries Science Center, La Jolla, CA]) was diluted to 25 ng/μL in filter-sterilized distilled water for subsequent PCR amplifications. Amino acids at positions 83, 292, and 299 from the *E. glacialis* rod opsin gene were inferred by PCR amplification of genomic DNA within conserved regions of exon 1 and exon 4 identified from a consensus sequence of aligned cetacean and bovine rod opsin cDNA sequences (National Center for Biotechnology Information [NCBI] IDs: AF055456.1 [*T. truncatus*], AF055314.1 [common dolphin, *Delphinus delphis*], AF055315.1 [long-finned pilot whale, *Globicephala melas*], AF055316.1 [Sowerby's beaked whale, *Mesoplodon bidens*], NM_001014890.1 [*B. taurus*]). The exon 1 upstream sense degenerate oligonucleotide primer containing position 83 was 5′ATGAAYGGGACGGARGGCCTGAACT 3′ and the exon 1 downstream antisense oligonucleotide primer was 5′CGCCCAGGGTGGCAAAGAAGCCCTC 3′. The exon 4 upstream sense oligonucleotide primer containing positions 292 and 299 was 5′GCAGCTGCCCAACAGCAGGAGTCGG 3′ and the exon 4 downstream antisense oligonucleotide primer was 5′CTGCTTGTTCATCATGATGTAGATG3′. Each amplification was carried out with the AmpliTaq Gold 360 Master Mix kit (Applied Biosystems, Foster, CA) and contained 25 μL AmpliTaq Gold 360 Master Mix, 22 μL filter-sterilized distilled water, 0.5 μM of each primer and 25 ng of genomic DNA. The cycling parameters were 95°C for 5 min followed by 35 cycles consisting of 50°C for 1 min, 72°C for 1 min, and 95°C for 1 min, with a final 8 min at 72°C.

Amino acids at positions 180, 277, 285, and 308 from the *E. glacialis* MWS cone opsin gene were inferred from gene nucleotide sequences obtained by PCR amplification of genomic DNA within conserved regions of exon 3 and exon 5. Amino acid substitutions were identified at the above positions from a consensus sequence of aligned cetacean and bovine M/LWS cone opsin cDNA sequences (NCBI IDs: AY228451.1 [*D. delphis*], AF055457.1 [*T. truncatus*], AY228446.1 [*G. melas*], AY228450.1 [harbor porpoise, *Phocoena phocoena*], NM_174566.1 [*B. taurus*]). The exon 3 upstream sense oligonucleotide primer containing position 180 was 5′-GGATCACCGGTCTCTGGTC-3′, and the exon 3 downstream antisense oligonucleotide primer was 5′-CCATCTTTGGCTGGAGCAG-3′. The exon 5 upstream sense degenerate oligonucleotide primer containing positions 277, 285, and 308 was 5′GTGGCRAAGCAGCAGAAAGA 3′, and the exon 5 downstream antisense degenerate oligonucleotide primer was 5′CTGSCGGTTCATRAAGAC 3′. PCR mixtures and cycling parameters were the same as described above except that the annealing temperature was 53°C.

PCR products were cloned into pCR 2.1 (Invitrogen, Carlsbad, CA) or sequenced directly using the exon specific primers described above. All sequencing was performed at the Tufts Core Facility (Boston, MA) by an ABI PRISM 3130XL DNA sequencer (AME Bioscience, Toroed, Norway) using LongTrace software (Nucleics, Bendigo, Australia). Recombinant clones were sequenced using plasmid specific m13 forward and reverse primers. Each opsin sequence presented here is based on consensus sequences of both the forward and reverse sequences resulting from at least two clones from two independent PCR amplifications from each of the two

E. glacialis individuals. Sequence alignments were made using Vector NTI (Invitrogen, Carlsbad, CA).

RESULTS

We have examined the *E. glacialis* rod and MWS cone opsin genes to determine the amino acids found at key positions in the opsin proteins involved in modulating the position of the absorbance maximum of each visual pigment. Previous work has shown that three amino acid substitutions in the rod opsin protein at positions 83 (exon 1), 292 and 299 (exon 4) and a single amino acid substitution in the MWS cone opsin protein at position 308 (exon 5) fully explain the differences in λ_{max} values between the cetacean rod and MWS cone visual pigments and the corresponding visual pigments from a terrestrial relative, *B. taurus* (Fasick and Robinson 1998). Figure 1 shows amino acid alignments of the *E. glacialis* sequences along with the rod and MWS cone opsin sequences from other cetaceans previously reported. The sequences from *B. taurus* have been included for reference to a terrestrial mammal. When these alignments are examined, we see that the *E. glacialis* rod opsin possesses

Rod

Exon 1, TM 2 **83**

Eubalaena glacialis	TVQHKKLRTPLNYILLNLAVA**N**LFMVFGGFTTTLYTSLHAYFVFGP
Bos taurus	TVQHKKLRTPLNYILLNLAVA**D**LFMVFGGFTTTLYTSLHGYFVFGP
Delphinus delphis	TVQHKKLRTPLNYILLNLAVA**N**LFMVFGGFTTTLYTSLHAYFVFGP
Globicephala melas	TVQHKKLRTPLNYIPLNLAVA**N**LFMVFGGFTTTLYTSLHAYFVFGP
Mesoplodon bidens	TVQHKKLRTPLNYILLNLAVA**N**LFMVLGGFTTTLYTSMHAYFIFGP
Tursiops truncatus	TVQHKKLRTPLNYILLNLAVA**N**LFMVFGGFTTTLYTSLHAYFVFGP

Exon 4, TM 7 **292** **299**

Eubalaena glacialis	AAAQQQESATTQKAEKEVTRMVIIMVVAFLICWLPYASVAFYIFIHQGSDFGPIFMTIP**A**FFAKSS**S**IYNPVIYIMMNKQ
Bos taurus	AAAQQQESATTQKAEKEVTRMVIIMVIAFLICWLPYAGVAFYIFTHQGSDFGPIFMTIP**A**FFAKTS**A**VYNPVIYIMMNKQ
Delphinus delphis	AAAQQQESATTQKAEKEVTRMVIIMVVAFLICWVPYASVAFYIFTHQGSDFGPIFMTIP**S**FFAKSS**S**IYNPVIYIMMNKQ
Globicephala melas	AAAQQQESATTQKAEKEVTRMVVIMVVAFLICWVPYASVAFYIFTHQGSNFGPIFMTIP**S**FFAKSS**A**IYNPVIYIMMNKQ
Mesoplodon bidens	AAAQQQESATTQKAEKEVTRMVVIMVVAFLICWVPYASVAFYIFTHQGSNFGPIFMTIP**S**FFAKSS**A**IYNPVIYIMMNKQ
Tursiops truncatus	AAAQQQESATTQKAEKEVTRMVIIMVVAFLICWVPYASVAFYIFTHQGSDFGPIFMTIP**S**FFAKSS**S**IYNPVIYIMMNKQ

MWS Cone

Exon 3, TM 4 **180**

Eubalaena glacialis	GITGLWSLAIISWERWMVVCQPFGNVRFDAKLAIAGIAFSWIW**A**AVWTAPPIFGWS
Bos taurus	GITGLWSLAIISWERWMVVCKPFGNVRFDAKLAITGIAFSWIW**A**AVWTAPPIFGWS
Delphinus delphis	GITGLWSLAIISWERWMVVCKPFGNVRFDAKLAIAGIAFSWIW**A**AVWTAPPIFGWS
Globicephala melas	GITGLWSLAIISWERWMVVCKPFGNVRFDAKLAIVGIAFSWIW**A**AVWTAPPIFGWS
Phocoena phocoena	GITGLWSLAIISWERWMVVCKPFGNVRFDAKLAIAGIAFSWIW**A**AVWTAPPIFGWS
Tursiops truncatus	GITGLWSLAIISWERWMVVCKPFGNVRFDAKLAIAGIAFSWIW**A**AVWTAPPIFGWS

Exon 5, TMs 6 and 7 **277** **285** **308**

Eubalaena glacialis	VAKQQKESESTQKAEKEVTRIVMVMIFT**Y**CLCWGPY**T**FFACFAAAHPGYAFHPLVAALP**S**YCAKSATIYNPIIYVFMN
Bos taurus	VAKQQKESESTQKAEKEVTRMVMVMIFA**Y**CLCWGPY**T**FFACFAAAHPGYAFHPLVAALP**A**YFAKSATIYNPIIYVFMN
Delphinus delphis	VAKQQKESESTRKVEKEVTRMVMVMIFA**Y**CLCWGPY**T**FFACFAAAHPGYAFHPLVAALP**S**YFAKSATIYNPIIYVFMN
Globicephala melas	VAKQQKESESTRKAEKEVTRMVMVMIFA**Y**CLCWGPY**T**FFACFAAAHPGYAFHPLVAALP**S**YFAKSATIYNPIIYVFMN
Phocoena phocoena	VAKQQKESESTRKAEKEVTRMVMVMIFA**Y**CLCWGPY**T**FFACFAAAHPGYAFHPLVAALP**S**YFAKSATIYNPIIYVFMN
Tursiops truncatus	VAKQQKESESTRKAEKEVTRMVMVMIFA**Y**CLCWGPY**T**FFACFAAAHPGYAFHPLVAALP**S**YFAKSATIYNPIIYVFMN

Figure 1. Alignment of amino acid sequences deduced from cetacean rod opsin exons 1 and 4 and MWS cone opsin exons 3 and 5. Amino acid substitutions associated with significant wavelength modulation are in bold and numbered. Underlined regions indicate transmembrane (TM) helices. The Genbank accession numbers for bovine (*B. taurus*), common dolphin (*Delphinus delphis*), right whale (*E. glacialis*), long-finned pilot whale (*Globicephalia melas*), Sowerby's beaked whale (*M. bidens*), and bottlenose dolphin (*T. truncatus*) rod visual pigments are K00502, AF055314, HM371330, AF055315, AF055316, and AF055456, respectively. The Genbank accession numbers for bovine (*B. taurus*), common dolphin (*Delphinus delphis*), right whale (*E. glacialis*), long-finned pilot whale (*Globicephala melas*), harbor porpoise (*P. phocoena*), and bottlenose dolphin (*T. truncatus*) M/LWS cone opsins are NM_174566, AY228451, HM371331, AY228446, AY228448, and AF055457, respectively.

Table 1. Amino acid substitutions in rod, M/LWS cone pigments and opsin mutants with resulting absorbance maxima (λ_{max}).

	Rod				M/ LWS cone			
	λ_{max} (nm)	83	292	299	λ_{max} (nm)	180	277	285	308
Mutants[a]									
B. taurus	501	D	A	S	552 (w.t.)	A	Y	T	A
	500 (w.t.)	D	A	A					
	499	N	A	S					
	495	N	A	A					
	489	N	S	S					
	487	D	S	A					
	485	N	S	A					
T. truncatus					552	A	Y	T	A
					524 (w.t.)	A	Y	T	S
Cetacea[a]									
E. glacialis[b]	499	N	A	S	524	A	Y	T	S
Megaptera novaeangliae	492	D	S	S					
T. truncatus	488	N	S	S	524	A	Y	T	S
Delphinus delphis	489	N	S	S					
G. melas	488	N	S	S	531[c]	A	Y	T	S
M. bidens	484	N	S	A					
P. phocoena					522[c]	A	Y	T	S

Note: Single and three letter amino acid nomenclature: D = Asp, N = Asn, A = Ala, S = Ser, Y = Tyr, T = Thr.

[a]Data from Fasick and Robinson 1998, 2000.

[b]Estimate from rod and LWS cone mutants.

[c]Data from Newman and Robinson 2005.

the amino acids ^{83}Asn, ^{292}Ala, and ^{299}Ser while the *E. glacialis* MWS cone opsin possesses ^{180}Ala, ^{277}Tyr, ^{285}Thr, and ^{308}Ser. When the combination ^{83}Asn, ^{292}Ala, and ^{299}Ser is incorporated into the bovine rod visual pigment mutagenesis model shown in Table 1, it results in a λ_{max} of 499 nm. This λ_{max} value is red-shifted from the experimentally determined values from the odontocetes (λ_{max} = 481–489 nm; McFarland 1971; Fasick *et al*. 1998; Fasick and Robinson 1998, 2000) as well as *M. novaeangliae* (λ_{max} = 492 nm; McFarland 1971) and is essentially identical to that of most terrestrial mammals including bovine (λ_{max} = 500 nm). The *E. glacialis* MWS cone visual pigment possesses the single amino acid substitution ^{308}Ser, previously shown to result in a dramatically blue-shifted λ_{max} of 524 nm in the dolphin MWS cone pigment when compared to the *B. taurus* LWS cone visual pigment (λ_{max} = 552 nm) while the amino acids ^{180}Ala, ^{277}Tyr, and ^{285}Thr are conserved in both the cetaceans as well as in bovine. Interestingly, *E. glacialis* appears to possess a typical blue-shifted, odontocete-like MWS cone visual pigment, but a terrestrial-like rod visual pigment.

Like all other cetaceans examined to date, *E. glacialis* possesses the ^{83}Asn substitution found in exon 2 of the rod opsin gene. However, unlike both the odontocetes and terrestrial mammals, *E. glacialis* possesses the ^{292}Ala and ^{299}Ser substitutions found in exon 4 of the rod opsin gene. The *E. glacialis* rod opsin exon 1 sequence (46 amino acids/138 nucleotides) containing ^{83}Asn shares the following amino acid/nucelotide percent identities: 93/97, *M. bidens*; 96/94, *B. taurus*; 98/98, *G. melas*; 100/98,

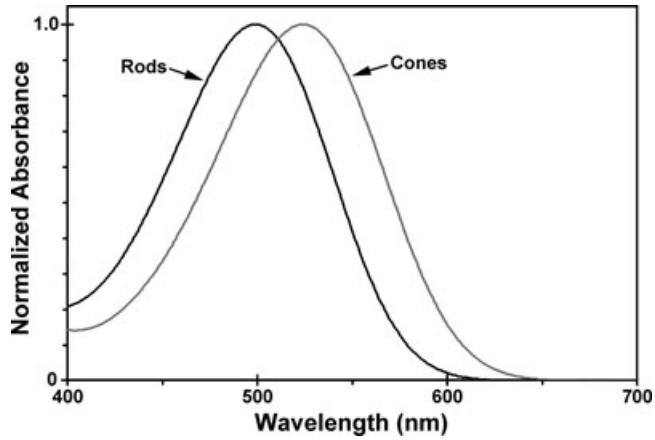

Figure 2. Estimated absorbance spectra of the rod and MWS cone visual pigments of *E. glacialis*. Absorbance spectra are positioned at values for the rod visual pigment (λ_{max} = 499 nm) and the MWS cone visual pigment (λ_{max} = 524 nm) estimated from the mutagenesis study described in Table 1. Absorbance spectra were generated using templates plotting both the rod and cone spectra normalized to their respective absorbance maxima from 400 to 700 nm (Stavenga *et al.* 1993).

D. delphis and *T. truncatus.* The *B. taurus* sequence was included for comparison to a closely related terrestrial mammal which possesses the amino acids associated with the ancestral rod opsin (Yokoyama 2008). It is clear that exon 1 of the cetacean rod opsin gene is highly conserved between species and that the amino acid substitution ^{83}Asn is found in both the mysticete and odontocete suborders. As seen in Figure 1, the *E. glacialis* rod pigment exon 4 sequence (80 amino acids/240 nucleotides), which includes amino acid positions 292 and 299, shares the following amino acid/nucelotide percent identities: 92/97, *M. bidens*; 92/91, *B. taurus*; 92/98, *G. melas*; 96/98, *D. delphis* and *T. truncatus.* Within the odontocetes, the delphinids all share both the ^{292}Ser and ^{299}Ser substitutions while *M. bidens,* a member of the Ziphiidae, differs in that it possesses the ^{292}Ser and ^{299}Ala substitutions. On this basis, the rod opsin sequences from exon 4 are not as well conserved amongst the cetaceans as those of exon 1. Many terrestrial mammals, like *B. taurus*, differ from the cetaceans in possessing the ^{292}Ala and ^{299}Ala substitutions. Unlike both the odontocetes and terrestrial mammals, *E. glacialis* possesses the ^{292}Ala and ^{299}Ser substitutions in exon 4. From these data we estimate that *E. glacialis* possesses a rod visual pigment with a λ_{max} value of 499 nm as depicted in Figure 2.

Although a single amino acid substitution (^{308}Ala $\rightarrow$ ^{308}Ser) in the cetacean MWS cone opsin protein fully explains the differences in λ_{max} values between the bovine LWS (^{308}Ala, λ_{max} = 552 nm) and delphinid MWS (^{308}Ser, λ_{max} = 524 nm) cone visual pigments, there are as many as four amino acid substitutions that provide significant contributions to λ_{max} modulations between the mammalian MWS and LWS cone visual pigments. These include amino acid substitutions at positions 180 in exon 3 and 277, 285, and 308 in exon 5 (Yokoyama 2008). Because the amino acids at these positions have been shown to cause the modulation between the λ_{max} of the mammalian MWS and LWS pigments, we have sequenced these two exons from *E. glacialis* genomic DNA to determine if amino acid substitutions occur at

these four positions among cetacean species. As seen in Figure 1, all odontocetes examined to date, as well as *E. glacialis*, possess identical amino acids at these four positions (^{180}Ala, ^{277}Tyr, ^{285}Thr, and ^{308}Ser) in their MWS cone opsin genes. When the sequences from exons 3 and 5 from *B. taurus* are included in this comparison, the only observed amino acid substitution is that of ^{308}Ala $\rightarrow$ ^{308}Ser, which results in the 28 nm blue-shift between the bovine LWS and cetacean MWS cone visual pigments. As seen in Figure 1, the *E. glacialis* MWS cone pigment exon 3 sequence (56 amino acids/168 nucleotides), which includes amino acid position 180, shares the following amino acid/nucelotide percent identities: 98/98, *P. phocoena*; 96/94, *Bos taurus*; 96/97, *G. melas*; 98/97, *D. delphis* and *T. truncatus*. The *E. glacialis* MWS cone pigment exon 5 sequence (78 amino acids/234 nucleotides), which includes amino acid positions 277, 285, and 308, shares the following amino acid/nucelotide percent identities: 95/96, *P. phocoena*; 95/93, *B. taurus*; 95/95, *G. melas*; 94/95, *D. delphis;* and 95/96, *T. truncatus*. Because *E. glacialis* possesses the identical amino acids as those found in the odontocetes at all four critical positions known to influence the absorbance maximum between the MWS and LWS cone visual pigments, we estimate that *E. glacialis* possesses a MWS cone visual pigment with a λ_{max} value of 524 nm as depicted in Figure 2.

Discussion

In order to estimate the λ_{max} values of the *E. glacialis* rod and MWS cone visual pigments, we have sequenced regions of the *E. glacialis* rod and MWS cone opsin genes in order to identify amino acid substitutions critical to λ_{max} modulation. After comparing amino acid substitutions in exons 1 and 4 from the rod opsin gene, we estimate that *E. glacialis* possesses a rod visual pigment with a λ_{max} of 499 nm based on the incorporation of the amino acids ^{83}Asn, ^{292}Ala, and ^{299}Ser. These specific amino acid substitutions, summarized in Table 1, were identified in earlier studies in which bovine/dolphin mutagenesis models determined the contribution made by both individual substitutions as well as combined substitutions. The λ_{max} of the *E. glacialis* rod visual pigment is essentially identical to values identified from terrestrial mammals, many of which possess the amino acid substitutions ^{83}Asp, ^{292}Ala, and ^{299}Ala, with λ_{max} values around 500 nm. Although the *E. glacialis* rod visual pigment appears to have an absorbance maximum similar to that of a terrestrial mammalian rod visual pigment, the *E. glacialis* rod visual pigment has achieved this absorbance maximum using the amino acid substitutions ^{83}Asn, ^{292}Ala, and ^{299}Ser. When we examine the odontocete rod visual pigments and the respective mutations constructed in the bovine rod opsin, the ^{292}Ala $\rightarrow$ ^{292}Ser substitution results in the strongest degree of blue-shift (12 nm between wild-type bovine and the Ala292Ser mutant) followed by the ^{83}Asp $\rightarrow$ ^{83}Asn substitution (4 nm between wild-type bovine and the Asp83Asn mutant). These two amino acid substitutions have occurred frequently during the evolution of the vertebrate rod visual pigments and have appeared on five separate occasions (four times in fish, once in mammals), causing similar functional changes each time (Yokoyama 2008). The ^{83}Asn substitution appears to be common in the mysticete whales, being recently identified in a study of nine other baleen whales, including members of the families Neobalaenidae and Eschrichtiidae as well as members of the genus *Balaenoptera*, that examined a 19 amino acid region of inferred sequences from exon 1 and showed that all but one of these species share ^{83}Asn, with the exception of *M. novaeangliae* possessing ^{83}Asp

(Sugawara *et al*. 2010). From this we can hypothesize that upon entering an aquatic photic environment the ancestral cetacean rod opsin gene underwent adaptive evolution utilizing the amino acid substitutions found in many present-day odontocetes, ^{83}Asp → ^{83}Asn, ^{292}Ala → ^{292}Ser, and ^{299}Ala → ^{299}Ser, resulting in rod visual pigments that are blue-shifted relative to their terrestrial counterparts. Based on this hypothesis, it appears that the mysticete whales, at least *M. novaeangliae* and *E. glacialis*, have undergone secondary adaptations as evidenced by the single reverse substitutions ^{83}Asn → ^{83}Asp and ^{292}Ser → ^{292}Ala occurring in *M. novaeangliae* and *E. glacialis*, respectively, resulting in rod visual pigments red-shifted for these two species relative to the cetacean ancestral state. Although *E. glacialis* has been observed to dive to considerable depths to forage, the species is typically classified as a surface forager, often skim-feeding on zooplankton in the top 2 m of the water column (Kenney *et al*. 2001). The spectral placement of the *E. glacialis* rod visual pigment λ_{max} around 500 nm appears then to be an adaptation to a photic environment associated with extremely shallow foraging depths where the rod photoreceptors, under scotopic light conditions, would function optimally.

Our estimation of the *E. glacialis* MWS cone visual pigment having a λ_{max} of 524 nm is supported upon examination of the amino acids involved in the wavelength modulation between the LWS and MWS cone visual pigments of other mammals. The human MWS cone visual pigment ($\lambda_{max} = 530$ nm) was formed by a duplication event of the ancient vertebrate LWS cone visual pigment ($\lambda_{max} = 560$ nm) opsin gene and the subsequent amino acid substitutions ^{180}Ser → ^{180}Ala, ^{277}Tyr → ^{277}Phe, ^{285}Thr → ^{285}Ala (Asenjo *et al.* 1994, Yokoyama 2008), while the blue-shifted λ_{max} associated with the mouse MWS cone visual pigment ($\lambda_{max} = 508$ nm) results from the amino acid substitution ^{308}Ala → ^{308}Ser (Sun *et al.* 1997). The amino acid composition at these four positions in the ancestral cetacean LWS cone visual pigment is found in *B. taurus* ($\lambda_{max} = 552$ nm) (Yokoyama 2008). By examining these four amino acid positions, we observe only the single amino acid substitution ^{308}Ala → ^{308}Ser which accounts for the entire 28 nm blue-shift between the ancestral (bovine) LWS ($\lambda_{max} = 552$ nm) and dolphin MWS ($\lambda_{max} = 524$ nm) cone visual pigments. Because *E. glacialis* shares the same amino acids at these four positions as the dolphin MWS cone visual pigment, we estimate that the *E. glacialis* MWS cone visual pigment possesses a λ_{max} of 524 nm. Previous work has shown that the delphinid MWS cone visual pigments range in λ_{max} from 524 nm in *T. truncatus* (Fasick *et al*. 1998) to 531 nm in *G. melas* (Newman and Robinson 2005), based on values determined from purified pigments resulting from *in vitro* expression of the MWS cone opsin coding regions. The MWS opsin coding regions (364 amino acids) from *T. truncatus* and *G. melas* share 99% amino acid identity and differ by a total of two amino acids with only a single conservative amino acid substitution (Ala171Val) occurring within a transmembrane region (TM 4). It is highly unlikely that such a conservative amino acid substitution in a helix positioned relatively distant from the chromophore would account for a 7 nm shift in λ_{max} values between these two MWS cone visual pigments. A more likely explanation for the differences associated with the λ_{max} values from the *T. truncatus* and *G. melas* MWS cone visual pigments is the variable absorbance scatter associated with these expressed pigments. Because the λ_{max} value of 524 nm from the *T. truncatus* MWS cone visual pigment results from an expressed pigment with far less scatter than that of the *G. melas* MWS cone visual pigment (see Fasick *et al*. 1998, Fasick and Robinson 1998, and Newman and Robinson 2005 for comparison), we have estimated the MWS cone visual pigment for *E. glacialis* to be 524 nm.

As with the rod opsin gene, we can speculate that upon entering an aquatic photic environment, the ancestral cetacean MWS opsin gene underwent adaptive evolution utilizing the single amino acid substitution ^{308}Ala $\rightarrow$ ^{308}Ser found in all cetaceans examined to date. However, unlike the rod opsin gene, there does not appear to be evidence for secondary adaptation within the mysticete MWS cone visual pigments. Of the four amino acid substitutions previously identified as key regulators of mammalian M/LWS cone visual pigment absorbance modulation, the only nonconservative amino acid substitution proximal to the chromophore binding pocket that has occurred in both the odontocetes and the mysticetes relative to their terrestrial ancestor is ^{308}Ala $\rightarrow$ ^{308}Ser.

The dynamic photic environment that cetaceans live in, specifically the wavelengths of visible light available for scotopic and photopic vision, may provide answers as to why we observe a broader range of λ_{max} values in the rod pigments when compared to the MWS cone pigments. Photopic or daytime vision requires enough photons to activate cone photoreceptor cells and is limited to relatively shallow depths near the air/water interface. Because all cetaceans must inhabit this surface photic environment to breathe, it is logical to predict that all cetacean MWS cone visual pigments would possess similar λ_{max} values. Alternatively, scotopic or dim-light vision requires far fewer photons to activate the rod photoreceptor cells and can occur in a wider range of depths. Here we observe a correlation between foraging depth and λ_{max} values: as foraging depth increases, λ_{max} values of the rod visual pigments decrease in order to match the blue-shifted photic environment. Thus, we observe highly conserved MWS cone opsin genes and MWS cone λ_{max} values adapted to the photopic light conditions encountered by all cetaceans at the air/water interface, and less conserved rod opsin genes resulting in a range of rod λ_{max} values adapted to specific scotopic light conditions associated with diverse foraging depths.

In summary, we have estimated the absorbance maxima of the *E. glacialis* rod and LWS cone visual pigments to be 499 nm and 524 nm, respectively. Unlike the odontocetes, which possess significant blue-shifting in both the rod and cone visual pigments, *E. glacialis* appears to possess significant blue-shifting only in the MWS cone pigment while the rod pigment exhibits an absorbance spectrum essentially identical to those of terrestrial mammals. As fresh tissue becomes available, it will be useful to confirm our results by expressing the *E. glacialis* visual pigments and directly measuring the absorbance spectra.

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