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Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

We thank B. Stephens for comments and suggestions on earlier versions of the manuscript. This work was supported by the NSF, NOAA and the International Geosphere Biosphere Program/Global Analysis, Interpretation, and Modeling Project. S.F. and J.S. were supported by NOAA's Office of Global Programs for the Carbon Modeling Consortium.

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Unsuspected diversity among marine aerobic anoxygenic phototrophs

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Aerobic, anoxygenic, phototrophic bacteria containing bacteriochlorophyll *a* (Bchl*a*) require oxygen for both growth and Bchl*a* synthesis[1–6]. Recent reports suggest that these bacteria are widely distributed in marine plankton, and that they may account for up to 5% of surface ocean photosynthetic electron transport[7] and 11% of the total microbial community[8]. Known planktonic anoxygenic phototrophs belong to only a few restricted groups within the Proteobacteria α-subclass. Here we report genomic analyses of the photosynthetic gene content and operon organization in naturally occurring marine bacteria. These photosynthetic gene clusters included some that most closely resembled those of Proteobacteria from the β-subclass, which have never before been observed in marine environments. Furthermore, these photosynthetic genes were broadly distributed in marine plankton, and actively expressed in neritic bacterioplankton assemblages, indicating that the newly identified phototrophs were photosynthetically competent. Our data demonstrate that planktonic bacterial assemblages are not simply composed of one uniform, widespread class of anoxygenic phototrophs, as previously proposed[8]; rather, these assemblages contain multiple, distantly related, photosynthetically active bacterial groups, including some unrelated to known and cultivated types.

Most of the genes required for the formation of bacteriochlorophyll-containing photosystems in aerobic, anoxygenic, phototrophic (AAP) bacteria are clustered in a contiguous, 45-kilobase (kb) chromosomal region (superoperon)[6]. These include *bch* and *crt* genes coding for the enzymes of the bacteriochlorophyll and carotenoid biosynthetic pathways, and the *puf* genes coding for the subunits of the light-harvesting complex (*pufB* and *pufA*) and the reaction centre complex (*pufL* and *pufM*). To better describe the nature and diversity of planktonic, anoxygenic, photosynthetic bacteria, we screened a surface-water bacterial artificial chromo-

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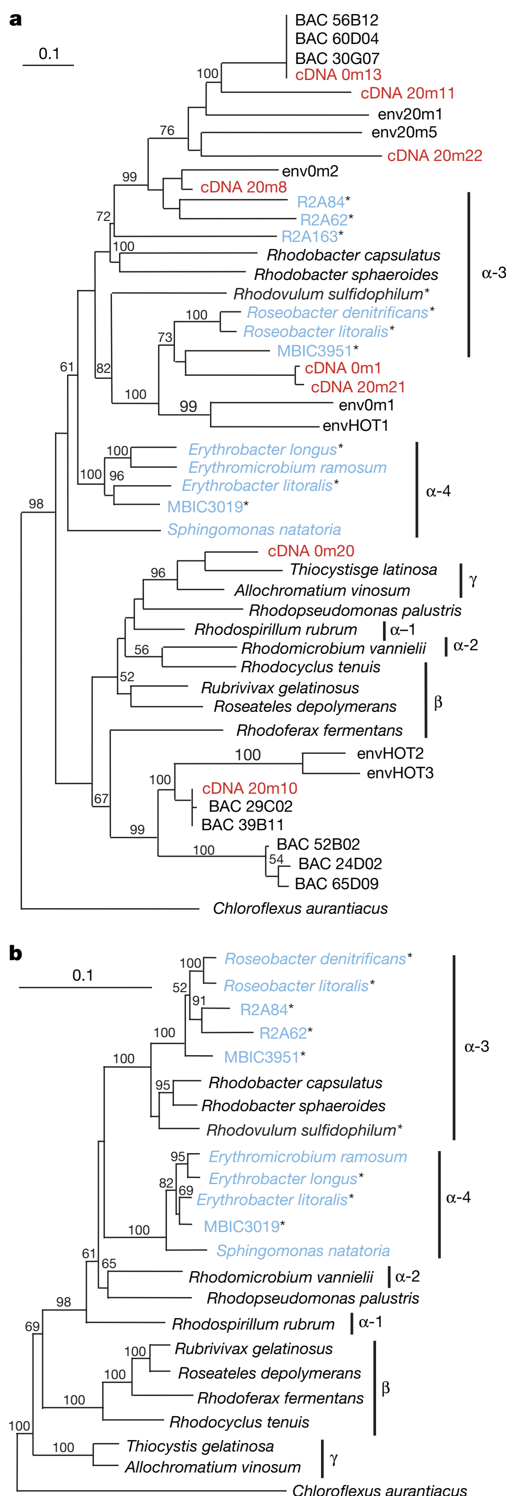

Figure 1 Phylogenetic relationships of *pufM* gene (**a**) and rRNA (**b**) sequences of AAP bacteria. **a**, **b**, Evolutionary distances for the *pufM* genes (**a**) were determined from an alignment of 600 nucleotide positions, and for rRNA genes (**b**) from an alignment of 860 nucleotide sequence positions. Evolutionary relationships were determined by neighbour-joining analysis (see Methods). The green non-sulphur bacterium *Chloroflexus aurantiacus* was used as an outgroup. *pufM* genes that were amplified by PCR in this study are indicated by the env prefix, with 'm' indicating Monterey, and HOT indicating Hawaii ocean time series. Cultivated aerobes are marked in light blue, bacteria cultured from sea water are marked with an asterisk, and environmental cDNAs are marked in red. Photosynthetic α-, β- and γ-proteobacterial groups are indicated by the vertical bars to the right of the tree. Bootstrap values greater than 50% are indicated above the branches. The scale bar represents number of substitutions per site.

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some (BAC) library[9] prepared from surface-water marine bacterioplankton, for clones containing *pufL* and *pufM*[10,11] genes.

Several *puf*-containing BAC clones were identified in screens using primers[10] designed to amplify almost the entire *pufL* and *pufM* region (1520–1580 nucleotides). In addition, several coastal α-proteobacterial strains previously isolated off the Oregon coast were also screened (R2A strains)[12]. The *pufM* phylogenetic tree (Fig. 1a) encompassed two principal clades, one containing α-3 and α-4 proteobacteria and one containing α-1, α-2, β- and γ-proteobacteria representatives, in agreement with an earlier study[10]. The BAC clones fell into three groups on the basis of *pufM* gene phylogeny (Fig. 1a). One group (BAC clones 30G07, 56B12 and 60D04) was most closely related to *pufM* from α-proteobacteria isolates R2A62 and R2A84 (ref. 12) (*Roseobacter*-like, Fig. 1b), and was placed in the group containing α-3 Proteobacteria. The other two groups (29C02, 39B11 and 24D02, 52B02, 65D09) branched together, and were most similar to the freshwater β-proteobacterium *Rhodoferax fermentans* (Fig. 1a, b). *puf* genes were also amplified by polymerase chain reaction (PCR) from DNA extracts of a mixed bacterioplankton assemblage, sampled at the same location as where the BAC library originated: Monterey Bay, California. These Monterey Bay *pufM* sequences clustered with BAC clones 30G07, 56B12 and 60D04, the α-proteobacteria isolates R2A62 and R2A84 (env0m2, env20m1 and env20m5), and the group composed of *Roseobacter* isolates (env0m1). The phylogenetic relationships of *pufM* genes were generally consistent with those determined from ribosomal RNA gene sequences, with the exception of the *pufM* cluster containing the α-1, α-2, β- and γ-proteobacteria (Fig. 1b).

To identify groups actively expressing photosynthetic genes in natural populations, we used PCR with reverse transcription (RT-PCR) to identify photosynthetic-operon messenger RNAs from the same environment. The *pufL*/*M* primer set failed to amplify any complementary DNA. However, a different primer targeting a smaller fragment (156 nucleotides) of the *pufM* gene revealed five different groups of *pufM* in a Monterey Bay *pufM* cDNA gene library (cDNAs in Fig. 1). One cDNA group clustered with the aerobic marine phototroph *Roseobacter denitrificans*, another with strain R2A84, and one was most similar to the *pufM* sequence from a γ-proteobacterium, *Thiocystis gelatinosa*. Two *pufM* cDNA groups clustered with the different BAC clones, implying that these BAC inserts originate from bacteria that were actively expressing photosynthetic genes.

To better characterize the bacteria from which these photosynthetic operons originated, the BAC inserts (29C02, 41 kb; 60D04, 103 kb; 65D09, 87 kb) were fully sequenced and the photosynthetic operons compared with those of cultured proteobacteria. The operon organization of BAC clone 65D09 (and 29C02; data not shown), which falls inside the α-1/α-2/β/γ *pufM* cluster, is considerably different from the operon organization of any previously reported photosynthetic organism (Fig. 2). Clones 65D09 and 29C02 are similar in their photosynthetic operon organization (data not shown), and when compared with both α- or β-proteobacterial (*Rhodobacter sphaeroides*[13] and *Rubrivivax gelatinosus*[14], respectively) operon organization, more closely resemble the β-proteobacterial operon (Fig. 2). BAC clone 60D04, which is related to α-3 proteobacteria (on the basis of *pufM* sequences), also differs significantly in gene arrangement from any other photosynthetic operon organization reported to date. It most closely resembles the photosynthetic operons from *Rhodobacter capsulatus*[8] and *R. sphaeroides*[13,15], in both organization and gene content (it contains the *bchIDO* and *crtD* genes, which are missing in *R. gelatinosus*[14] and BAC clones 29C02 and 65D09). The superoperonal gene arrangement, *crtEF–bchCXYZ–puf* and *bchFNBHLM–lhaA–puhA*[13–17], found in *R. sphaeroides, R. capsulatus* and *R. gelatinosus* are conserved among the naturally occurring planktonic bacterial genomes (Fig. 2), suggesting that the photosynthetic apparatus in the bacteria from which the BACs originated are functional.

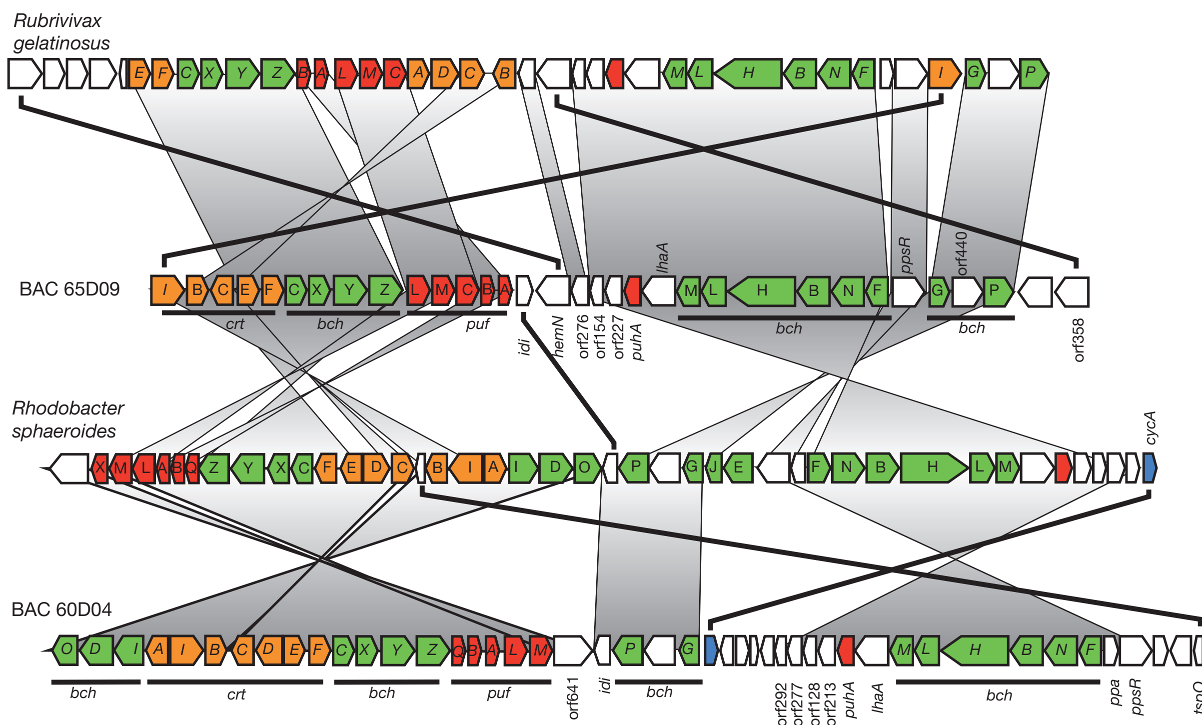

Figure 2 Schematic comparison of photosynthetic operons from *R. gelatinosus* (β-proteobacteria), *R. sphaeroides* (α-proteobacteria) and uncultured environmental BACs. ORF abbreviations use the nomenclature defined in refs 13, 14 and 24. Predicted ORFs are coloured according to biological category: green, bacteriochloropyll biosynthesis genes; orange, carotenoid biosynthesis genes; red, light-harvesting and reaction centre genes; and blue, cytochrome c_2. White boxes indicate non-photosynthetic and hypothetical proteins with no known function. Homologous regions and genes are connected by shaded vertical areas and lines, respectively.

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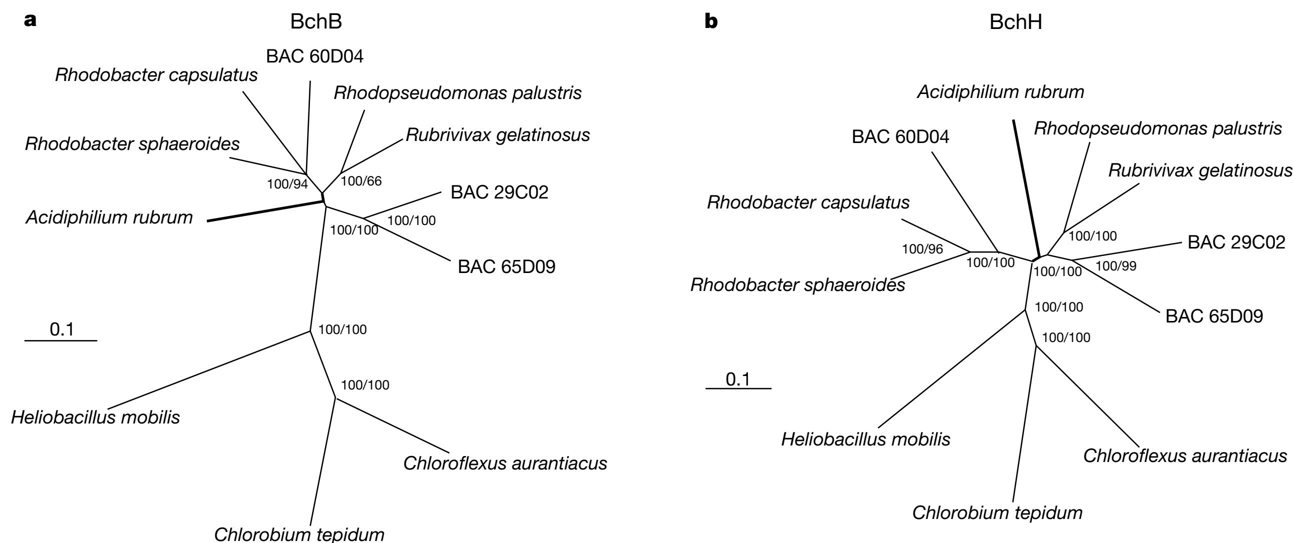

Figure 3 Phylogenetic analyses of BchB and BchH proteins. **a**, Phylogenetic tree for the BchB protein. **b**, Phylogenetic tree for the BchH protein. The BchH sequences from *Chlorobium vibrioforme*[25] and BchH2 and BchH3 from *C. tepidum*[18] were omitted from the tree because these genes potentially encode an enzyme for bacteriochlorophyll *c* biosynthesis and are probably of distinct origin (J. Xiong, personal communication). Bootstrap values (neighbour-joining/parsimony method) greater than 50% are indicated next to the branches. The scale bar represents number of substitutions per site. The position of *Acidiphilium rubrum* (bold branch) was not well resolved by both methods.

We compared several photosynthetic genes found on the oceanic bacteriochlorophyll superoperons with characterized homologues from cultured photosynthetic bacteria. The relationships among Bchl*a* biosynthetic proteins BchB (a subunit of light-independent protochlorophyllide reductase) and BchH (magnesium chelatase) were determined[18] (Fig. 3). Similar to *pufM* relationships, BchB and BchH proteins from BAC clone 60D04 were most similar to homologues from *R. capsulatus* and *R. sphaeroides* (Fig. 3). Bchl*a* biosynthetic proteins from BAC clones 29C02 and 65D09 were most closely related to those of *R. gelatinosus* (no sequences are yet available from a photosynthetic γ-proteobacterium) and *Rhodopseudomonas palustris*, again in agreement with the phylogenetic relationships of the *pufM* sequences (Fig. 1).

Recent analyses suggest possible horizontal transfer of the photosynthetic gene cluster in purple bacteria[14], and this possibility complicates definitive identification of the organismal origins of these operons based solely on photosyntethic gene analysis. However, gene assignment of open reading frames (ORFs) revealed that more than 75% of ORFs outside the photosynthetic superoperon on BAC 65D09 are most similar to proteins from γ-proteobacteria, whereas ORFs from BAC clone 60D04 are most similar to those of α-proteobacteria. The phylogenetic assignments on the basis of *puf* gene similarities and arrangement are therefore consistent with the chromosomal context external to the respective photosynthetic superoperons in the BAC clones analysed.

In this study, no sequences recovered in Monterey Bay waters were similar to those of *Erythrobacter* species—one of the more commonly cultured Bchl*a*-containing bacteria recovered from the open ocean[8]. We therefore used the same *pufM* primers on bacterioplankton DNA extracts from waters of the central North Pacific Ocean (Hawaii ocean time series station[19]; envHOT clones in Fig. 1). One HOT environmental *pufM* group (represented by envHOT1 clone) clustered with sequences from α-3 proteobacteria isolates, and another group (envHOT2 and envHOT3) clustered with the BAC sequences related to the freshwater β-proteobacterium, *R. fermentans*. The results suggest that similar groups involved in oceanic aerobic anoxygenic photosynthesis are found in surface waters from both neritic and oceanic systems. These groups do not appear to be similar (at least with respect to their photosynthetic operon) to cultivated *Erythrobacter* species.

Recently, it has been suggested that cultivated *Erythrobacter* species (α-4 subclass of proteobacteria) may represent the predominant AAPs in the upper ocean[8,20]. Surprisingly, we were not able to retrieve photosynthetic operon genes belonging to this group in any of the samples analysed. Furthermore, very few sequences related to *Erythrobacter* species have been reported in 16S rDNA clone libraries constructed from marine plankton DNA, also suggesting that this group may not represent the predominant AAPs in the upper ocean. Some of the representative AAPs we found were related to cultured marine bacteria (*Roseobacter* and *Roseobacter*-like bacteria within the α-3 subclass of proteobacteria). Members of this group have also been frequently retrieved in 16S rRNA clone libraries and represent a large proportion of bacterioplankton rDNAs in coastal waters[21]. However, other groups (found in both BAC and cDNA libraries) were only distantly related to known anoxygenic phototrophs, and have never before been observed in marine plankton. This implies that in addition to *Erythrobacter* and *Roseobacter* species, other yet-to-be-cultivated bacteria (most likely related to β- or γ-proteobacteria) actively participate in oceanic aerobic, bacteriochlorophyll-based photosynthesis. The discovery of new marine AAP bacteria through culture-independent genomic analyses emphasizes the complementary nature of culture-based and cultivation-independent approaches, which taken together provide a much more comprehensive perspective than either does alone. Our new observations should help direct current efforts aimed at characterizing those microbes responsible for oceanic, bacteriochlorophyll-mediated photosynthesis, a newly recognized[7,8] but poorly understood process in marine plankton. □

Methods

BAC library and environmental cDNA preparation

The surface-water BAC library construction has been described previously[9] and was prepared from sea water from station M2 (located approximately 45 km offshore of Moss Landing, California) pre-filtered through a GF/A glass fibre filter (approximate particle size less than 1.6 μm) to remove cell aggregates and larger eukaryotic phytoplankton cells. Sea water from the HOT station (22.4° N, 158.0° W) was collected on 17 March 1998. For preparation of environmental cDNA, sea water was collected from 0 m and 20 m on 26 April 2000 at station 4B off Moss Landing, California (36.77° N, 122.02° W) aboard the RV *Western Flyer*, and picoplankton were collected onto Sterivex Cartridges (Millipore) and

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stored as described previously[21]. After the initial treatment with proteinase K and SDS described by ref. 22, total RNA from 100 μl of the lysate from the Sterivex cartridges were further purified using the RNeasy tissue kit (Qiagen) and the protocol for lysed cells. To further remove DNA from the RNA preparations, the samples were treated by the DNAfree kit (Ambion) following the manufacturer's protocol. cDNAs were synthesized by reverse transcription using 2 μl of the RNA extracts using random hexamers as primers, using the TaqMan RT kit (Applied Biosystems) according to the manufacturer's protocol. Genomic DNA contamination of cDNA preparations was examined by 5′-nuclease assays comparing gene copies in cDNA preparations and in controls with no reverse transcriptase added. These control assays tested for the presence of both rRNA[21] and protein-encoding genes (M. T. Suzuki *et al*., unpublished results). In both assays we observed no signal in controls without reverse transcriptase.

The annotated BAC insert sequences are available at the Monterey Bay Coastal Ocean Microbial Observatory site at http://www.tigr.org/tdb/MBMO/.

pufL and *pufM* amplification

Primers used in this study were pufL, forward (5′-CTKTTCGACTTCTGGGTSGG-3′)[10]; pufM, reverse (5′-CCATSGTCCAGCGCCAGAA-3′) (a modification of the primer reported by ref. 10); and pufM, forward (5′-TACGGSAACCTGTWCTAC-3′).

Phylogenetic analysis

pufM sequences from the current study combined with sequences from public databases were translated and the protein sequences aligned using the pileup program of the Wisconsin package (GCG). DNA sequences and protein alignments were imported into a database using ARB software (http://www.arb-home.de). We aligned DNA sequences based on the protein alignment. Evolutionary distances were calculated with the dnadist program of the PHYLIP package[23], using the Kimura 2-parameter model. Phylogenetic trees were inferred using the neighbour program of the PHYLIP package. To evaluate the reliability of the branching patterns, 100 random bootstrap re-samplings were performed using the program seqboot, with subsequent phylogenetic analyses performed as above. Ribosomal RNA phylogenetic analyses were performed as described above, on alignments encompassing 860 nucleotide sequence positions. For analysis of the shorter *pufM* cDNA sequences obtained through RT-PCR, a neighbour-joining tree was imported into the ARB database, and the cDNAs were added to tree using the ARB_PARSIMONY program, without local optimization and using a mask that included only those positions encompassing the cDNA sequences.

Received 23 August; accepted 4 December 2001.

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Acknowledgements

We thank S. Haddock for computational advice; J. Zehr and D. Karl for the HOT samples; the captains and crew of the RV *Point Lobos* and *Western Flyer* for assistance at sea; and M. Heaney, V. Sapiro, S. Lo, and M. Holmes for database and software support. Preliminary sequence data for *R. palustris* was obtained from the DOE Joint Genome Institute (JGI) at http://www.jgi.doe.gov/JGI_microbial/html/index.html. This research was supported by the National Science Foundation (J.F.H, J.A.E and E.F.D.) and the David and Lucile Packard Foundation (E.F.D).

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to E.F.D. (e-mail: delong@mbari.org). The sequences reported in this study are deposited with GenBank under accession numbers AB018690, AB027515, AF393983–AF393991, AF393993-AF394002, AY044244–AY044250 and AE008919–AE008921.

Sex differences in emigration and mortality affect optimal management of deer populations

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Populations of red deer that are limited by food, like those of many other ungulates[1–3], commonly include more females than males[4–7]. We assessed the contribution of variation in sex- and age-specific rates of mortality and emigration to density-dependent changes in the adult sex ratio, using long-term observations and demographic experiments involving the red deer population on Rum, Scotland[4,5]. We incorporated these effects in a stochastic model of local populations under different management regimes to show here that, when female numbers are allowed to increase to more than 60% of the ecological carrying capacity, the sustainable annual harvest of males from local deer populations will fall. Because males are typically culled by fee-paying hunters and generate more income than females[5,8,9], income will decrease as the male harvest falls. Because numbers of female deer throughout much of the Highlands probably exceed the threshold at which male density starts to be affected[5], many managers might be able to raise income from local deer populations by reducing female numbers, with potential benefits to the vegetation of Scottish Highland environments[10].

Populations of red deer (*Cervus elaphus* L.) throughout the Scottish Highlands include roughly twice as many females aged ≥1 yr as males (Fig. 1a). To investigate the consequences of increasing population density for males and females, in 1972 we released the red deer population of the 12-km^2 north block of Rum

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