We used methods to house and feed male C57BL/6 mice, a commonly used model in aging research with an average life span of ~30 months, were recently described [T. D. Pugh, T. D. Oberley, R. Weindruch, Cancer Res. 59, 642 (1999)].

Total RNA was extracted from frozen tissue by using TRIZOL reagent [Life Technologies]. Polyadenylate (poly(A)competent) RNA was purified by using a T7 MegaScript Kit (Ambion) with 1.5 μg/mL of poly(A)* DNA and poly(A)T oligo(dT)25 (Genset). After second-strand synthesis, the reaction mixture was extracted with phenol–chloroform–isoamyl alcohol and, after ethanol precipitation, the RNA was dissolved in water. Hybridization solutions were removed and the gene capsule, a keratin-like matrix that embeds the mitochondria (3), and became fully soluble in 6 M guanidine–hydrochloride.

Two-dimensional polyacrylamide gel electrophoresis (PAGE) was used to separate proteins. Proteins were visualized by staining with Coomassie brilliant blue R-250 or silver staining (5). The detected protein spots were excised and digested with trypsin or other proteases as indicated in the figure legends. Mass spectrometry was performed to identify the proteins. The proteins were subjected to MALDI–TOF mass spectrometry and analyzed by using the SpectrumMill database.

The selenoprotein PHGPx was purified from rat sperm mitochondria by using a combination of centrifugation, chromatography, and electrophoresis. The purified protein was digested with trypsin and the resulting peptides were analyzed by mass spectrometry. The identified protein sequence matched the sequence of PHGPx as determined by sequence analysis of cDNA clones.

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region were detected (Fig. 1C, left lane). Protein immunoblotting (10) revealed that the most prominent band reacted with PHGPx antibodies (Fig. 1C, right lane). NH₂-terminal sequencing (11) of the 21-kD band (46% of total protein content according to stain intensity) revealed that it consisted of at least 95% pure PHGPx. We therefore investigated the composition of the mitochondrial capsules by two-dimensional (2D) electrophoresis (12) (Fig. 2A) followed by microsequencing (13) or matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis (14) for identification (Fig. 2B). The spot migrating with an apparent molecular mass of 21 kD and focusing at a pH near 8 (spot 3) proved to be PHGPx, according to the masses of tryptic peptides detected by MALDI-TOF spectrometry (Fig. 2B). All tryptic fragments yielding MALDI-TOF signals of high intensity could be attributed to PHGPx or trypsin. The predicted NH₂-terminal (positions 3 to 12) and COOH-terminal peptides (positions 165 to 170), the fragment corresponding to positions 100 to 105, and those expected from the basic sequence part (residues 119 to 151) were too small to be reliably identified. The fragment corresponding to positions 34 to 48 comprising the active site selenocysteine was not detected either. The more acidic spot 4 of Fig. 2A, the more basic spots 1, 2, and 5, and those exhibiting a smaller apparent molecular mass (spots 6 and 7) also contained PHGPx (15). Spots 1 to 6 were essentially homogeneous. Spot 7 showed a trace of impurity that could not be identified by masses of fragments. Integrated stain intensities of the individual spots indicate that PHGPx constituted about 50% of the capsule material.

Minor components present in the gel (Fig. 2A, spots 10 to 13) were assigned to mitochondrial proteins or to cytosolic contaminations. Spots 8 and 9 consisted of "outer dense fiber protein," a cysteine-rich structural sperm protein that is associated with the helix of mitochondria in the midpiece but also extends into the flagellum. SMCP was not detected. This basic protein that becomes superficially associated with the outer mitochondrial membranes in late spermatids and epididymal spermatozoa (5) might have been degraded by trypsination during capsule preparation.

PHGPx was enzymatically inactive in mature spermatozoa prepared from the tail of the epididymis and was not reactivated by the reduced form of glutathione (GSH) in the low

Fig. 1. Presence of PHGPx in the mitochondrial capsule of rat spermatozoa. (A) Mitochondrial capsule prepared by trypsination and centrifugation (4, 9). The same preparation as shown in (A) but after exposure to 0.1 M 2-mercaptoethanol for 15 min at 4°C. Contamination of the capsule material by mitochondria is evident from the presence of mitochondrial ghosts (arrowhead). Scale bars, 0.3 μm. (C) SDS-PAGE of proteins extracted from capsule material by treatment with 0.1 M 2-mercaptoethanol, 0.1 M tris-HCl (pH 7.5), and 6 M guanidine-HCl. Left lane is stained with colloidal gold; right lane demonstrates presence of PHGPx by protein immunoblotting (10).

Fig. 2. Analysis of the composition of the mitochondrial capsule of spermatozoa. (A) Two-dimensional electrophoresis of purified dissolved capsule material. Proteins were focused in a linear pH gradient from 3 to 10 (horizontal direction), then reduced, amidocarboxymethylated, subjected to SDS electrophoresis, and stained with Coomassie blue. MALDI-TOF analysis of the visible spots identified the following proteins (NCBI database): spots 1 to 7, PHGPx (accession number 544434); spots 8 and 9, outer dense fiber protein (accession number P21769); spots 10 and 11, voltage-dependent anion channel-like protein (accession number 540011); spot 12, "stress-activated protein kinase" (accession number 493207); spot 13, glycerol-3-phosphate dehydrogenase (accession number P35571) (22). (B) MALDI-TOF spectrum (overview) of tryptic peptides obtained from PHGPx as found in spot 3. Abscissa, mass/charge ratio (m/z) of the peptide fragments; ordinate, arbitrary units of intensity (a.i.). The insert lists the mass signals 1 to 17 attributed to tryptic fragments of PHGPx with measured m/z values and corresponding residues in the PHGPx sequence. Peaks 8 and 11 correspond to tryptic fragments with oxidized methionine residue; peak 15 corresponds to a fragment with an NH₂-terminal pyroglutamyl residue. T, trypsin-derived fragments.

Fig. 3. Formation of PHGPx-containing aggregates by H₂O₂ in the absence of GSH. (A) Whole rat spermatozoa were solubilized with 0.1 M 2-mercaptoethanol and 6 M guanidine-HCl and freed from low molecular weight compounds as described (17). Aliquots of the protein mixture (0.05 mg of protein) were subjected to SDS-PAGE under reducing (lanes 1 and 2) and nonreducing conditions (lanes 3 and 4) at zero time (lanes 1 and 3) or after 15-min exposure to 75 μM H₂O₂ (lanes 2 and 4). PHGPx-containing bands were detected by protein immunoblotting (10). (B) Lanes 1 to 4 show the same experiments but performed with purified rat testis PHGPx. A band of dimerized PHGPx was observed in lane 4.
millimolar range, as used under conventional test conditions (16). High concentrations of thiols (0.1 M 2-mercaptoethanol or DTT), which in the presence of guanidine fully dissolved the capsule, generated a substantial PHGPx activity (17). In fact, the specific activities thereby obtained from mitochondrial capsules (5600 ± 290 mU/mg protein) exceeded, by a factor of 20, the values measured in spermatogenic cells from testicular tubules (250 ± 10 to 260 ± 10 mU/mg). The latter observation is consistent with the expression of PHGPx as active peroxidase in round spermatids (with the expression of PHGPx as active peroxidase in round spermatids (18)). The latter observation is consistent with the expression of PHGPx as active peroxidase in round spermatids (18).

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References and Notes
10. Spermatozoa of 4-month-old Wistar rats were collected by squeezing the cauda epididymis and were defrosted in phosphate-buffered saline (PBS) and centrifuged at 600g for 10 min. Spermaticogenic cells were prepared as described [M. L. Meistrich, J. Londer, W. A. Brock, S. R. Grimes, M. L. Mace, Biol. Reprod. 25, 1065 (1981)]. Sperm mitochondrial capsule was prepared according to (4).
11. Proteins were blotted onto nitrocellulose, probed with an antigen-purified rabbit antibody raised against pig heart PHGPx, and detected by biotinylated antibody to rabbit immunoglobulin G (IgG) and streptavidin alkaline phosphatase complex.
12. Before NH2-terminal sequencing, proteins were blotted onto polyvinylidene difluoride membranes for 16 hours at pH 8.3 (25 mM tris- HCl, 192 mM glycine) and stained with ninhydrin to visualize protein bands.
13. Mitochondrial caps (100 mg) were dissolved in 400 µl of a solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 20 mM tris base, and 0.5% IGP buffer (Pharmacia) and focused in an IEF-electrophore with an IPG-phor (Pharmacia) at 20°C by stepwise increasing voltage up to 5000 V, followed by further focusing for 30 min. The pH gradient was nonlinear from 3 to 10 or linear from 3 to 10 or 6 to 11. The focused IGP strips were equilibrated for SDS-PAGE with a solution containing 50 mM DTT in 6 M urea, 0.05 M tris-HCl buffer (pH 8.8), and thereafter with the same buffer containing 250 mM iodoacetamide instead of DTT. The gels were then dried with Coomasie blue.
14. Protein spots from 1.5-mm 2D gels were digested with modified trypsin (Promega, sequencing grade) in 25 mM (NH4)2HCO3 overnight at 37°C. The digests were extracted twice, dried, and reconstituted in 10 µl of water. Peptides were first hydrolyzed on a reversed-phase capillary column (0.5 mm × 150 mm) with a gradient of acetonitrile in 0.1% formic acid/4 mM ammonium acetate at a flow rate of 5 µl/min. Aliquots of 5 µl were spotted onto Biobeads-treated glass fiber filters and sequenced on an Applied Biosystems 494A sequencer with standard pulsed liquid cycles.
15. Masses of tryptic fragments covering the positions of the PHGPx sequence 21 to 33, 49 to 59, 106 to 118, and 141 to 151 were identified by MALDI-TOF analysis in spots 1 to 7 (Fig. 2A). Sequence coverage was about 75%. Recovery of fragments comprising residues 3 to 9 and 165 to 170 (COOH-terminus) varied between spots. Five distinct spots in the 20-kD region were also separated using a thicker 2D gel developed with a nonlinear gradient from pH 3 to 10. Here, the presence of PHGPx was verified by sequencing major tryptic peptides. Again, the five spots representing PHGPx were the most abundant in the gel. The chemical modifications of PHGPx leading to distinct differences in charge and size were not elucidated. Sequencing revealed an identical NH2-terminus of the peptide isomers starting from position E149 Ser (1996). Tryptic peptides extending toward the COOH-terminus up to position 164 were also observed with the faster migrating species. Charge heterogeneity may arise from phosphorylation [R. Schuckelt et al., Free Radical Res. Commun. 14, 343 (1991)], deaminations of Gln and Asn residues, COOH-terminal degradation, and oxidation or elimination of selenium. The alternate roles of PHGPx as a glutathione-dependent hydroperoxide reductase or a structural protein are not necessarily related. A feature common to all glutathione peroxidases is a selenocysteine residue, which, together with a tryptophan and a glutamine residue, forms a catalytic triad (19). Therein the selenocysteine residue is oxidized by hydroperoxides with high rate constants. The reaction product, a selenocysteinic acid derivative, R-SeOH, reacts with GSH to form a selenol sulfide bridge between enzyme and substrate, R-Se-S-G, from which the ground-state enzyme is regenerated by a second GSH.
20. Mitochondrial capsules (5600 ± 290 mU/mg protein) but not in spermatogenic cells from testicular tubules (250 ± 10 to 260 ± 10 mU/mg). The latter observation is consistent with the expression of PHGPx as active peroxidase in round spermatids (18).
21. Formation of a structural element of the spermatozoon that is pivotal for male fertility. This reaction product, a selenenic acid derivative, R-SeOH, reacts with GSH to form a selenol sulfide bridge between enzyme and substrate, R-Se-S-G, from which the ground-state enzyme is regenerated by a second GSH. In analogy, PHGPx, which is the least specific of the glutathione peroxidases (19), can use protein thiols as alternate substrates to create protein aggregates that are cross-linked by selenol sulfide or disulfide bonds. This likely occurs when cells are exposed to hydroperoxides at low concentrations of GSH, as is documented for late stages of spermatogenesis (20).
22. Proteins derived from epididymal spermatozoa, when exposed to H2O2 in the absence of GSH, yielded a variety of PHGPx-containing aggregates (Fig. 3A). This process depends on the presence of thiol groups in proteins distinct from PHGPx, because under identical conditions only a marginal aggregate formation was observed with pure PHGPx (Fig. 3B).
23. Our findings require a fundamental reconsideration of the role of selenium in male fertility. The predominance of the selenoprotein PHGPx in the male reproductive system (3) has been believed to reflect the necessity to shield germ line cells from oxidative damage by hydroperoxides (3, 20). This concept still merits attention with regard to the mutagenic potential of hydroperoxides and probably holds true for the early phases of spermatogenesis. At this stage, phenomena attributed to the enzymatic activity of PHGPx or other glutathione peroxidases—for instance, silencing lipoxigenases, dampening the activation of nuclear factor kB, or inhibiting apoptosis (21)—may also be relevant. Mature spermatozoon, however, depend on PHGPx as a structural protein, because the morphological midpiece alterations that are observed in selenium deficiency likely result from impaired biosynthesis of the selenoprotein. In consequence, it is not the antioxidant capacity of PHGPx but the ability to use hydroperoxides for the formation of a structural element of the spermatozoon that is pivotal for male fertility.
was measured at 37°C with phosphatidylcholine hydroperoxide at 3 mM GSH according to (16). Control samples were treated identically but with 5 mM 2-mercaptoethanol.


22. In other gels, mitochondrial glutathione S-transferase subunit Yb-2 (accession number 121719) and endothelin converting enzyme (NCBI accession number 170656) could be identified by MALDI-TOF or peptide sequencing.

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Eutrophication, Fisheries, and Consumer-Resource Dynamics in Marine Pelagic Ecosystems

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Anthropogenic nutrient enrichment and fishing influence marine ecosystems worldwide (1). Alteration of resource availability represents a “bottom-up” perturbation of marine ecosystems, whereas removal of consumer biomass through fishing represents a “top-down” disturbance. An understanding of how bottom-up and top-down processes influence the dynamics of marine communities is necessary for effective management of marine ecosystems in the face of environmental variability and multiple human impacts. However, it is difficult to determine the effects of resource availability and food-web interactions in open (pelagic), highly variable marine systems; most propositions are based on anecdotal evidence from catastrophic events such as El Niño years (2), fishery collapses (3), and the introduction of exotic species (4). To determine how marine pelagic ecosystems respond to variation in the quantity of resources and consumers, I conducted meta-analyses of data from a variety of experimental and natural systems and examined whether changes in the abundance of consumers (pelagic zooplanktivorous fish) cascade down marine food webs to affect lower trophic levels, and whether changes in nutrient availability and primary productivity cascade up marine food webs to affect higher trophic levels.

To address these questions, I assembled data from experimental manipulations conducted in marine mesocosms and from long-term monitoring of open marine ecosystems. Experiments conducted in mesocosms eliminate open-system dynamics but represent controlled alterations of nutrient availability and food-web structure. In contrast, long-term monitoring of open marine systems documents patterns at realistic spatial and temporal scales. The first data set comprised phytoplankton and mesozooplankton (mostly herbivorous copepod crustaceans larger than 150 to 300 μm) data from marine mesocosm experiments where nutrient availability was manipulated by adding N compounds, or where food-web structure was manipulated by adding or removing zooplanktivorous fish or invertebrates (5). The second data set consisted of time series (7 to 45 years) of N availability (measured as the annual loading or as the average N concentration during winter months), primary productivity, and the biomass of phytoplankton, mesozooplankton, and pelagic zooplanktivorous fish for 20 open marine ecosystems (6).

For the mesocosm experiments, I quantified responses of phytoplankton and mesozooplankton to nutrient and food-web manipulations by using the natural logarithm of the ratio between the mean value of the variable in mesocosms with carnivores (zooplanktivorous fish or invertebrates) or nutrients (inorganic N compounds) added and in unmanipulated, control mesocosms (7). Zooplanktivores caused significant decreases in mesozooplankton biomass, both in mesocosms with no N added (Fig. 1A) and in mesocosms enriched with N (Fig. 1B). Zooplanktivores caused an increase in phytoplankton biomass, but this trend was statistically significant only in systems that were also enriched with N (Fig. 1, A and B). Nitrogen addition caused similar and significant increases in phytoplankton biomass in mesocosms containing two (phytoplankton and zooplankton; Fig. 1C) or three trophic levels (phytoplankton, zooplankton, and zooplanktivores; Fig. 1D). Under either food-web configuration, nutrient addition did not affect mesozooplankton biomass (Fig. 1, C and D). The effects of the manipulations were not significantly correlated with either experiment duration or mesocosm size in zooplanktivore-manipulation experiments (8), and the effects were only weakly correlated with duration but not with size in nutrient-manipulation experiments (9). Therefore, these results are unlikely to be biased by the short duration or small mesocosm sizes used in most experiments.

For the 20 open marine ecosystems, I examined the cross-correlation between time series of nutrients, productivity, and biomass of different trophic levels using Spearman rank correlation (10). Theoretical models exploring the relations among resource availability, food-web structure, and biomass of different trophic levels predict patterns of biomass accrual along productivity gradients at equilibrium, that is, after transient effects have disappeared (11, 12). Because seasonal events such as upwelling and sudden increases in fish density from immigration or spring reproduction are transient effects, I used yearly values of productivity and biomass to approximate equilibrium conditions. Year-to-year fluctuations in mesozooplankton biomass were negatively correlated with zooplanktivorous fish (r = −0.22; 95% confidence limits = −0.31 and −0.12; N = 19), indicating that fish predation may control mesozooplankton biomass. In contrast, the correlation between mesozooplankton and