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# Resistance to Genotoxic Stresses in *Arctica islandica*, the Longest Living Noncolonial Animal: Is Extreme Longevity Associated With a Multistress Resistance Phenotype?

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Bivalve molluscs are newly discovered models of successful aging. Here, we test the hypothesis that extremely long-lived bivalves are not uniquely resistant to oxidative stressors (eg, *tert*-butyl hydroperoxide, as demonstrated in previous studies) but exhibit a multistress resistance phenotype. We contrasted resistance (in terms of organismal mortality) to genotoxic stresses (including topoisomerase inhibitors, agents that cross-link DNA or impair genomic integrity through DNA alkylation or methylation) and to mitochondrial oxidative stressors in three bivalve mollusc species with dramatically differing life spans: *Arctica islandica* (ocean quahog), *Mercenaria mercenaria* (northern quahog), and the Atlantic bay scallop, *Argopecten irradians irradians* (maximum species life spans: >500, >100, and ~2 years, respectively). With all stressors, the short-lived *A i irradians* were significantly less resistant than the two longer lived species. *Arctica islandica* were consistently more resistant than *M mercenaria* to mortality induced by oxidative stressors as well as DNA methylating agent nitrogen mustard and the DNA alkylating agent methyl methanesulfonate. The same trend was not observed for genotoxic agents that act through cross-linking DNA. In contrast, *M mercenaria* tended to be more resistant to epirubicin and genotoxic stressors, which cause DNA damage by inhibiting topoisomerases. To our knowledge, this is the first study comparing resistance to genotoxic stressors in bivalve mollusc species with disparate longevities. In line with previous studies of comparative stress resistance and longevity, our data extends, at least in part, the evidence for the hypothesis that an association exists between longevity and a general resistance to multiplex stressors, not solely oxidative stress. This work also provides justification for further investigation into the interspecies differences in stress response signatures induced by a diverse array of stressors in short-lived and long-lived bivalves, including pharmacological agents that elicit endoplasmic reticulum stress and cellular stress caused by activation of innate immunity.

**Key Words:** *Arctica islandica*—Bivalves—Comparative biology—Endoplasmic reticulum stress—Longevity—Oxidation—Stress resistance.

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WITHIN the class *Bivalvia*, maximum life span differs more than 200-fold, more than any other noncolonial animal group (from in excess of 500 years (1) to less than 2 years) (2). This natural variation of life span makes them ideal model organisms to test predictions of major hypotheses of aging (3–15). Using the burrowing clam *Arctica islandica* (ocean quahog), which is the longest lived of all noncolonial animal species on earth (maximum species life span: >500 years) (1,7,8,16), we recently demonstrated that in bivalve models of extreme longevity there is an association between maximum species life-span potential and resistance to oxidative stress–induced mortality and also a marked resistance to oxidative stress–induced apoptotic cell death (17).

Originally studies in the invertebrate model organism *Caenorhabditis elegans* have suggested that antiaging genetic mutations extend life span by simultaneous activation of multiple cellular pathways that protect the tissues and organs from multiple forms of injury (18). Longevity evolved independently many times in various phyla and a wide range of comparative studies is needed to prove that expression of a multistress resistance phenotype is a key mechanism of successful aging, which is conserved among these various long-lived groups (19). The hypothesis that the ability to develop resistance to multiple stressors is associated with life span has been supported, at least in part, by recent studies showing that an association exists between superior cellular stress resistance and increased species longevity in insects (20–22), rodents (23–29), bats (30), and birds (31). These discoveries led to the development of unifying multiplex stress resistance models of aging (18,32). These models imply that an organism's ability to resist a multitude of environmental and cytotoxic stressors is associated with enhanced life span (33). It is yet to be determined whether this model is valid for bivalves. It has been hypothesized that maximal life spans of benthic communities (such as species of clam) evolve in relation to long-term cycles in benthic communities (34), which exert selective pressure for longevities somewhat longer than the cycle affecting recruitment. In support of this, long periods of failed recruitment have been reported for populations of *A islandica* (1). Under such evolutionary pressures, somatic maintenance will be favored more than reproductive output and one would expect to see enhanced cellular defense and repair mechanisms in those species (35). Although bivalves are unlikely to be exposed to the toxicants used in this study, their comparative resistance to these stressors is indicative of their ability to resist stressors and repair damage.

This study was designed to test the hypothesis that extremely long-lived molluscan species are not uniquely resistant to oxidative stressors but exhibit a multistress resistance phenotype. To test this hypothesis, we contrasted resistance to genotoxic stresses and to mitochondrial oxidative stressors in three bivalve mollusc species with dramatically differing life spans. We tested whether resistance to genotoxic stresses is associated with exceptional longevity because recent studies raise the possibility that DNA repair efficiency associates with life span in a diverse group of mammals (including muroid rodents (28,36), bats, and primates; Podlutsky and Austad, unpublished data, 2009). Moreover, a decline in DNA repair capacity has been linked to premature aging, cancer, and short life span in multiple models of aging. In this study, we focused on *A islandica* and the taxonomically related burrowing clam, *Mercenaria mercenaria* (northern quahog), which lives in a similar environment, has similar physiology, but has a shorter life span (maximum species life span: >100 years, Table 1). Recent studies have characterized several aspects of *A islandica* and *M mercenaria* physiology, which render these species especially useful models for aging research (1,6,17,37,38). It has been documented that both clam species used in this study are exceptionally long lived (1,13,38) and that *A islandica* demonstrates negligible rates of aging, little age-related decline in antioxidant capacity, nor increase in macromolecular damage (7), and an increased resistance to cellular stress than its shorter lived relative (17). Although early studies investigating the exceptional longevity of bivalves implicated superior antioxidant status (7), this is now not believed to be the case (35). Here, we test the hypothesis that extremely long-lived molluscan species are not uniquely resistant to oxidative stressors but exhibit a multistress resistance phenotype with longer lived species having evolved superior defense and repair mechanisms. We contrasted multistress resistance in the aforementioned species with that in the short-lived Atlantic bay scallop (*Argopecten irradians irradians*;

Table 1. Chronological Age, Maximum Reported Life Span, and Physiological Characteristics of the Marine Bivalve Species Used in This Study

| Species | Common Name | Average Chronological Age (y) | Maximum Life Span (y) | Maximum Size (mm) | Growth Rate (K; VBGF) | Mortality Rate (Z) | Age at Maturity (y) | Lifestyle | References |
|---|---|---|---|---|---|---|---|---|---|
| *Artica islandica* | Ocean quahog, mahogany clam | ~20 | >500 | 118 | 0.02 | 0.03 | 7–14 | Infaunal burrower | 16, 83 |
| *Mercenaria mercenaria* | Northern quahog, hard clam | ~8 | 106 | 150 | 0.210 | 1.32 | 2–5 | Infaunal burrower | 3, 17, 84 |
| *Argopecten irradians irradians* | Atlantic bay scallop | ~1 | 2 | 60 | n/a | 1.41 | 1 | Active swimmer | 85 |

*Note:* n/a = not applicable; VGBF = von Bertalanffy growth function.

maximum species life span: 2 years, Table 1). We assessed organismal mortality in response to both mitochondrial oxidative stressors and genotoxic DNA damaging agents, including topoisomerase inhibitors and agents that cross-link DNA or impair genomic integrity through DNA alkylation or methylation.

## Methods

### *Clam Collection and Maintenance*

The extremely long-lived ocean quahog (*A islandica*), the long-lived northern quahog (*M mercenaria*), and the short-lived bay scallop (*A i irradians*) were used in this study. Maximum species life span and physiological characteristics for each species are shown in Table 1. All clams used in this study were collected in July 2011 in the coastal waters of New England. In the coastal waters of New England, where these specimens were collected from *A islandica*, for *M mercenaria* and *A i irradians* are estimated to have maximum life spans of 220, 106, and 2 years, respectively (38–40). The clams were transported to the Marine Aquatic Resources Center of the Marine Biological Laboratory (Woods Hole, MA), where they were kept at constant temperature (12°C for *A islandica*, 20°C for *M mercenaria* and *A i irradians*, which typically live in warmer water than *A islandica*) in 500-L tanks for more than 1 week prior to the studies.

### *Studies on Stress Resistance*

For each experimental condition, 10–15 individuals of each species (*A islandica*, *M mercenaria*, and *A i irradians*) were placed in 10-L plastic aquaria containing fresh sea water. Periodically, aquaria were checked to observe siphons, which indicated that the stressors were inhaled. Additionally to ensure infiltration of the stressors, a section of the ventral margin of the shell of both clam species (*A islandica* and *M mercenaria*) was carefully removed, minimizing damage to tissues, using a handheld rotary saw. This prevented the animals sealing themselves off from the external environment, which they can do for prolonged periods of time (1). To induce mitochondrial oxidative stress, animals were treated with paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride; 1 mmol/L), which induces reactive oxygen species (ROS) production in mitochondria, or rotenone (25 µmol/L), which inhibits complex I and generates ROS in mitochondria. A range of genotoxic DNA damaging agents were used during the investigation. Topoisomerases participate in cellular processes associated with separation of DNA strands such as replication, transcription, recombination, and repair, and inhibitors of topoisomerases ultimately induce DNA breaks. To induce genotoxic stress by inhibition of the topoisomerase, we used the topoisomerase II poisons etoposide (0.1 mg/L) and camptothecin (50 nmol/L), a cytotoxic quinoline alkaloid, which inhibits topoisomerase I preventing DNA religation and therefore causes DNA damage and apoptosis. We also used epirubicin (1 mg/L), which acts by intercalating into DNA strands, which results in formation of complexes inhibiting DNA and RNA synthesis. It also triggers DNA cleavage and promotes the generation of ROS that cause cell and DNA damage. Mechlorethamine hydrochloride (nitrogen mustard, 100 µmol/L) was used as an alkylating agent that acts by binding to DNA, cross-linking two strands, and preventing cell duplication. We also tested the affects on organismal survival of methyl methanesulfonate (MMS; 200 µmol/L), which methylates DNA, stalling replication forks, and mitomycin C (100 nmol/L) as well as cisplatin (1 mg/L), which are potent DNA cross-linkers. To study organismal resistance to mitochondrial oxidative stress and genotoxic stresses, the survival of *A islandica*, *M mercenaria*, and *A i irradians* exposed to the aforementioned drugs was recorded for up to 15 days. During the stressor exposure, *M mercenaria* and *A i irradians* were held at 20°C, whereas *A islandica* were held at 10°C. The optimum concentrations of each chemical used were selected on the basis of a review of the literature and lethal doses used in previous in vivo and in vitro investigations with other species. Tanks were checked regularly and death was recorded when the bivalve shell "gaped" (opened because their adductor muscles no longer functioned) and did not respond to external stimuli.

### *Data Analysis*

Survival curves were compared using the logrank test in GraphPad Prism 4.0 software. $p < .05$ was considered statistically significant. Data are expressed as means ± *SEM*, unless otherwise indicated (41–44).

## Results

To assess resistance to mitochondrial oxidative stress, we obtained survival curves of the clams in the presence of rotenone and paraquat. In the case of both paraquat (Figure 1A) and rotenone (Figure 1B), *A islandica* were significantly the most resistant to mortality, as compared with both *M mercenaria* ($p < .0001$) and *A. i. irradians* ($p < .0001$). *Mercenaria mercenaria* were significantly more resistant to mortality than *A i irradians* ($p < .0001$). *Argopecten irradians irradians* were particularly susceptible to both, paraquat and rotenone, with all specimens dead after 1 and 2 days, respectively.

Next, we assessed organismal resistance to the genotoxic stressors epirubicin (Figure 2A), etoposide (Figure 2B), and camptothecin (Figure 2C). No mortality was observed in *M mercenaria* after 15 days exposure to any of the three stressors and again *A i irradians* was significantly least resistant to all three stressors ($p < .0001$ with all comparisons and stressors). In the case of epirubicin and etoposide, *A islandica* was less resistant than *M mercenaria* ($p < .0001$ with both stressors), but with camptothecin, no significant difference in survival was observed between the two species after 15 days of exposure ($p = .15$).

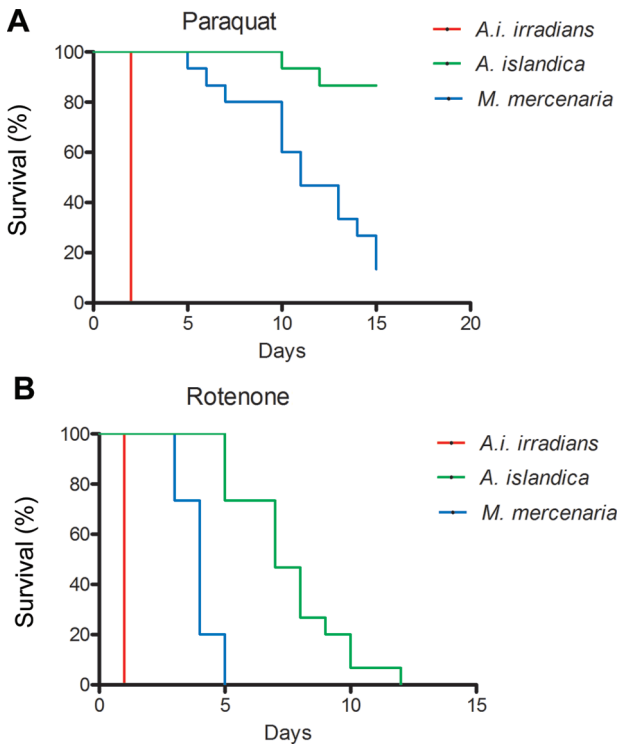


Figure 1. Survival analysis of *Argopecten irradians irradians*, *Mercenaria mercenaria*, and *Artica islandica* underexposure to the mitochondrial oxidative stressors paraquat (1 mmol/L; **A**) and rotenone (25 µmol/L; **B**).

We also assessed organismal resistance to a second set of genotoxic drugs, including mitomycin C (Figure 3A), cisplatin (Figure 3B), nitrogen mustard (Figure 3C), and MMS (Figure 3D). For any of these DNA damaging agents, *A i irradians* was again significantly less resistant to mortality than the two longer lived species ($p < .0001$, in all cases). There was no significant difference in survival rates of *A islandica* and *M mercenaria* when exposed to cisplatin ($p = .0881$), whereas *M mercenaria* was significantly more resistant to mortality induced by mitomycin C than the longer lived *A. islandica* ($p < .05$). However, in the case of the other two genotoxic agents, *M mercenaria* was significantly less resistant to mortality than the longer lived *A islandica* (MMS, $p < .0001$; nitrogen mustard, $p < .05$).

To summarize, we exposed the three species of bivalves to nine different stressors. With all stressors, the short-lived *A i irradians* were significantly less resistant to mortality than the two longer lived species, *M mercenaria* and *A islandica*. *Arctica islandica* were consistently more resistant than *M mercenaria* to mortality induced by mitochondrial oxidative stressors as well as to mortality induced by the DNA methylating agent nitrogen mustard and the DNA alkylating agent MMS. In case of the genotoxic DNA cross-linking agents mitomycin and cisplatin, *M mercenaria* were the most resistant species. In contrast, *M mercenaria* tended to be more resistant to the DNA intercalating agent epirubicin and the genotoxic stressors etoposide and campthotecin, which cause DNA damage by inhibiting topoisomerases.

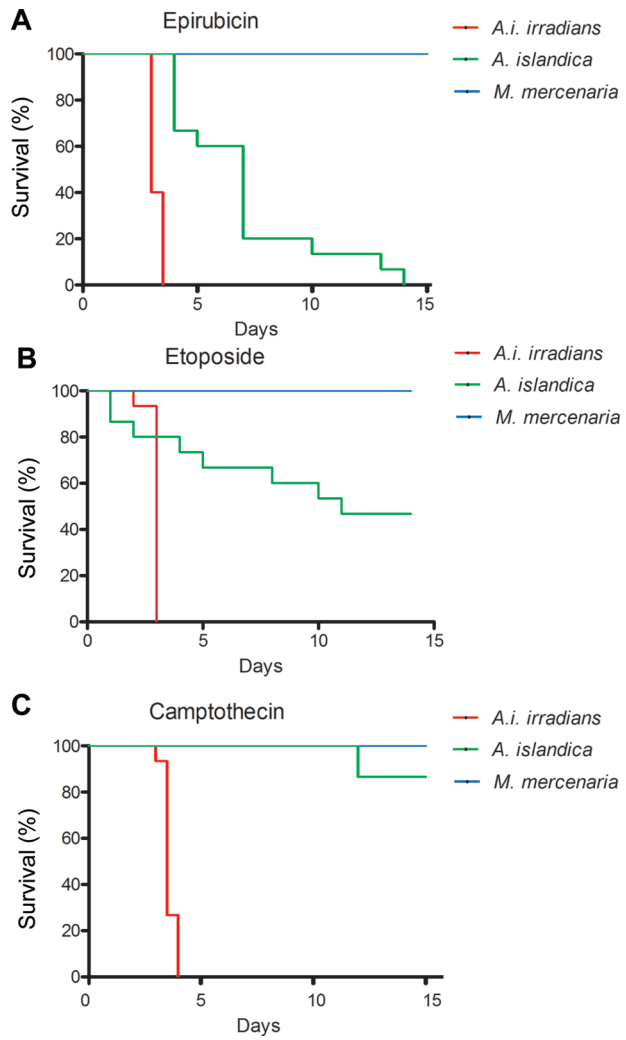


Figure 2. Survival analysis of *Argopecten irradians irradians*, *Mercenaria mercenaria*, and *Artica islandica* underexposure to the DNA intercalating agent, epirubicin, the topoisomerase II inhibitor etoposide, and the topoisomerase I inhibitor, camptothecin: (**A**) 1 mg/L epirubicin, (**B**) 0.1 mg/L etoposide, and (**C**) 50 nmol/L camptothecin.

## Discussion

Accumulating empirical data obtained in diverse vertebrate species and invertebrate model organisms suggest that resistance to the aging process is often reflected in resistance to oxidative stressors both at the cellular and organismal level (17,45,46). The first interesting finding in this study is that in bivalve models of extreme longevity, we documented an association between species life span and organismal resistance to the mitochondrial oxidative stressors paraquat and rotenone (Figure 1A,B). These findings are in accordance with the conclusions of our recent studies documenting an association between longevity and resistance to mortality induced by the oxidative stressor *tert*-butyl hydroperoxide and also marked cellular resistance to *tert*-butyl hydroperoxide stress–induced apoptotic cell death in long-lived *A islandica*, as compared with the shorter lived *M mercenaria* (17) and *A i irradians* (Ungvari,

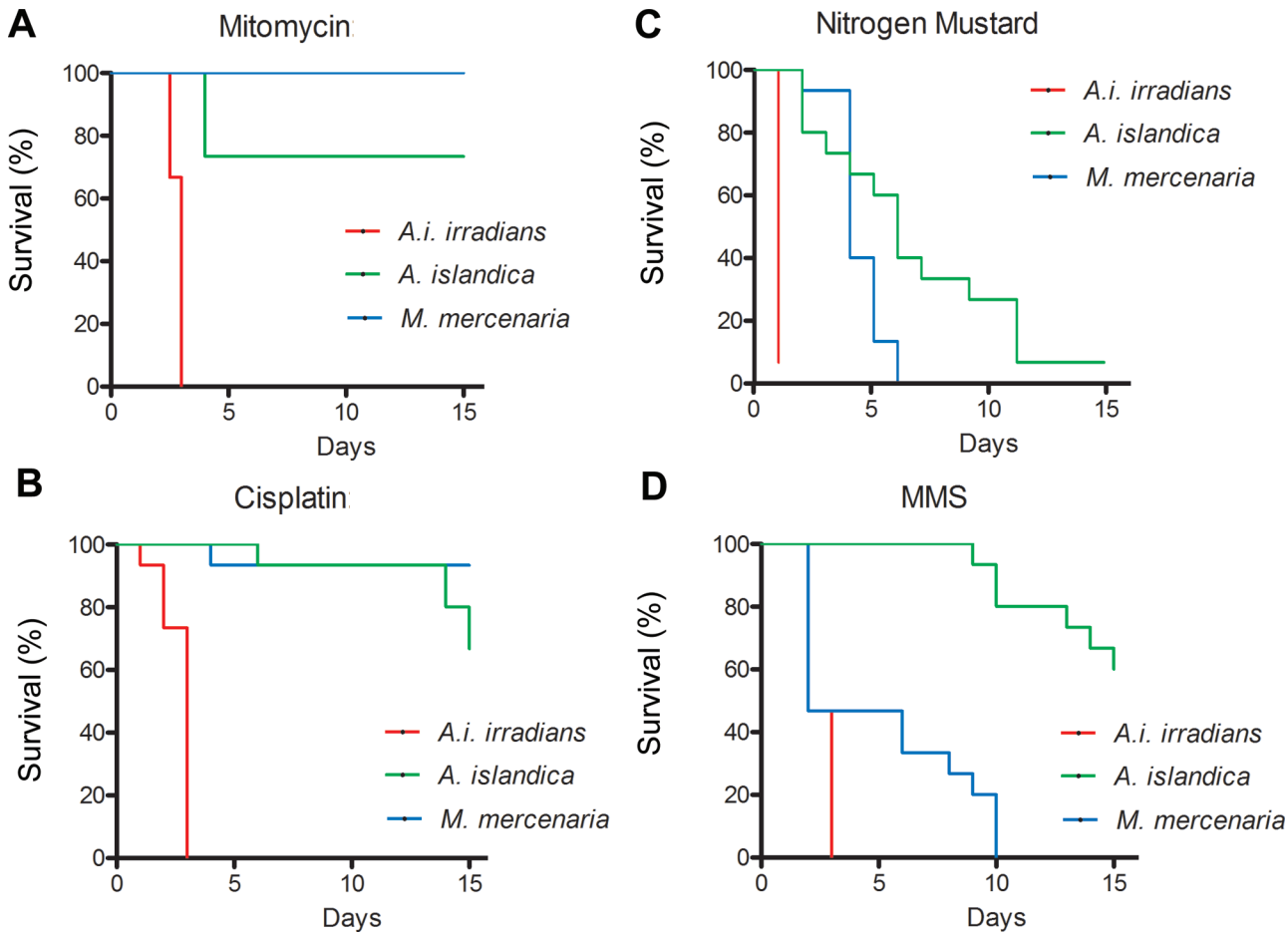


Figure 3. Survival analysis of *Argopecten irradians irradians*, *Mercenaria mercenaria*, and *Artica islandica* underexposure to the DNA cross-linking agents, mitomycin and cisplatin, the DNA alkylating agent, nitrogen mustard, and the DNA methylating agent, methyl methanesulfonate (MMS): (**A**) 100 nmol/L mitomycin, (**B**) 1 mg/L cisplatin, (**C**) 100 µmol/L nitrogen mustard, and (**D**) 200 µmol/L MMS.

Csiszar, and Ridgway, unpublished data, 2011). These findings also extend previous observations in a wide variety of experimental settings, ranging from invertebrate model organisms to laboratory rodents and primate fibroblasts (23,45–47). Recent findings in genetically manipulated laboratory mice (48–58) question the classical interpretation of the oxidative stress theory that increased levels of ROS per se play a key role in the aging process (59,60). Yet, the remarkable correlation between longevity and the increased oxidative stress resistance phenotype of longer lived animals in evolutionarily distant phyla is consistent with the existence of evolutionarily highly conserved pathways involved in both cellular oxidative stress resistance and life-span regulation, providing support for the oxidative stress hypothesis of aging.

The mechanisms underlying the increased cellular resistance to mitochondrial oxidative stress in long-lived bivalves are likely multifaceted. Previous studies demonstrate that differences in the efficiency of cellular antioxidant systems likely do not explain the superior oxidative stress resistance of *A islandica* (6,17). Recent studies also suggest that *A islandica* also does not exhibit a more pronounced homeostatic antioxidant response than shorter lived clams (17). In that context, it is interesting to note that in mice with overexpression or genetic knockout of major mitochondrial antioxidant enzymes (including MnSOD, catalase, and glutathione peroxidase), there is also no correlation between alterations of cellular antioxidant capacity and life span (48,50). Maintenance of protein homeostasis is also thought to be a critical determinant of both cellular stress resistance and life span (30,61), thus, further studies are warranted to determine whether in bivalves extreme longevity and resistance to oxidative stressors is, at least in part, due to enhanced protein recycling activities (17). Interestingly, resistance to mitochondrial oxidative stress in *A islandica* appears to associate with a low metabolic rate (at the temperatures used in these experiments respiration rates of 0.1, 0.7, and 1.0 mL $O_2$/g/h for *A islandica* (62), *A i irradians* (40), and *M mercenaria* (63), respectively, have been observed). Thus, further studies are warranted to test whether an association between basal metabolic rate and resistance to mitochondrial oxidative stressors exists in a range of bivalve species (eg, in long-lived

clam species living in tropical waters, such as the giant clam *Tridacna derasa*, which was reported to exhibit resistance to organic peroxide) (64).

We tested the hypothesis that in extremely long-lived molluscan species, resistance to mitochondrial oxidative stressors is associated with a multistress resistance phenotype. We focused on genotoxic stress resistance, as there is strong evidence that DNA mutations, DNA damage, and chromosomal abnormalities increase with age in mammals (65–68). Moreover, the existing data demonstrate an inverse relationship between DNA repair capacity and age in a number of experimental settings. Importantly, there is evidence supporting the view that in mammalian species a correlation exists between efficiency of DNA repair mechanisms (nucleotide excision repair, poly [ADP-ribose] polymerase [PARP] activity) and species life-span potential (69–72). Recent studies also demonstrate that long-lived *C elegans* mutants are resistant to DNA damaging agents and ultraviolet irradiation, likely due to genetically determined increases in nucleotide excision repair capacity (73). As we predicted, with all genotoxic stressors tested, the short-lived *A i irradians* were significantly less resistant to mortality than the two longer lived species, *M mercenaria* and *A islandica* (Figure 2 and Figure 3). The high susceptibility of *A i irradians* to genotoxic stressors could relate to intense proliferation rates in the animals compared with the clams such as *A islandica* (9). In vitro treatment of cultured mammalian cells with various genotoxic agents induces apoptosis within days by activating signaling events that couple DNA damage to the mitochondrial pathway of apoptosis (74). Because the greatest portion of mortality occurred in *A i irradians* over a relatively short period, future studies should also investigate interspecies differences in activation of the mitochondrial apoptotic pathway in response to DNA damage.

The data presented here suggests that both species of bivalves with a maximum life-span potential over a century have evolved regulated cellular processes to minimize the accumulated DNA damage or mutation. Further work is now required to investigate how DNA repair mechanisms differ between short-lived and long-lived species and how these are associated with incidences of neoplasias in species of bivalve. To date, only a few primary proliferative diseases (eg, sarcoma) have been reported in bivalves (75–77). The Registry of Tumours in Lower Animals contains a single report of gonadal neoplasia in *A islandica* (75), however, due to the lack of research effort, few reliable incidence statistics are available. Future work should also seek to define the relationship between cellular resistance to genotoxic stresses and expression and/or activity of proteins (eg, p53, PARP-1) that participate in DNA damage response pathways and to proteins that control DNA damage–induced changes in cell cycle regulation.

Contrary to our predictions, comparison of resistance to genotoxic stresses in *A islandica* and *M mercenaria* provides contrasting results. Although the longest living species, *A islandica*, was more resistant to mortality induced by the DNA methylating agent, nitrogen mustard, and the DNA alkylating agent, MMS, than the relatively shorter living *M mercenaria* (Figure 3C,D), the same trend was not observed for genotoxic agents that act through cross-linking DNA (Figure 3A,B). Furthermore, we found that *M mercenaria* tended to be more resistant to epirubicin and to genotoxic stressors, which cause DNA damage by inhibiting topoisomerases (Figure 2). Taken together, the aforementioned data do not fully support the validity of the hypothesis that in relatively long-lived burrowing clams a close association exists between longevity and a general resistance to multiplex stressors. Interestingly, although previous studies in mammals observed a general association between increased cellular resistance to multiplex stresses and longevity, they also observed incidences where the longer lived species did not exhibit greater resistance to all stressors (78). For example, cells from the long-lived naked mole rat are more sensitive than mouse cells to $H_2O_2$, ultraviolet light, rotenone, and to pharmacological agents that elicit endoplasmic reticulum stress (including tunicamycin and thapsigargin) (24). Further, cells of long-lived bat species are resistant to gamma-irradiation but are more sensitive to ultraviolet irradiation than cells of the shorter lived *M musculus* (Dr. Andrej Podlutsky, personal communication 2009). It is a likely explanation for the inconsistent pattern of differential stress resistance in the aforementioned cases that the different stress responsive and DNA repair pathways are not closely coordinated. We predict that the relative role of cellular DNA repair mechanisms and other stress resistance pathways in the evolution of longevity in the different animal groups depends on the relative importance of the different types of stress-induced macromolecular damages in the aging process and pathophysiology of these species.

*Limitations of the Study*

Although we have knowledge of the relationship between age and size in the species studied, we did not ascribe age to each of the animals used. Yet, all animals in the study could be classified as young sexually mature adults below the maximum asymptotic size attained for each of the species. Although all animals were deemed sexually mature, we did not control for sex or reproductive status, however, a review of the literature provides little evidence of any indication of gender effects on susceptibility to stress in bivalves (79,80). Additionally at Ocean Sciences, Bangor University, we have analyzed the ages of more than 1,000 individuals from a specific population of *A islandica* and found no significant difference in longevity between the sexes (unpublished data).

Future studies should also seek to use a greater range of species and investigate cellular pathways involved in resistance to genotoxic stresses, including DNA repair pathways. Although no continuous cell line has been

developed from marine bivalves and efforts to establish stable primary tissue cell cultures have been problematic, recent work demonstrates stable hemocyte cell cultures could be maintained for up to 1 week. Using such cell cultures, assessment of cellular DNA repair efficiency using the comet assay is technically feasible. Additionally, future studies should monitor toxicant concentrations in the seawater during the exposure periods to understand the role of differential uptake of the toxicants attributed to interspecies differences in respiratory anatomy and ventilation rate on the observed mortalities.

We have used the quinone-binding site inhibitor rotenone to inhibit complex I, which is known to significantly increase superoxide production rates during forward electron transport (81). Although rotenone is a useful tool in laboratory studies to test resistance to mitochondrial oxidative stress, it also interferes with ATP production, which may importantly contribute to its toxic effect in vivo. Because the lipophilic rotenone is easily taken up through the gills, it is highly toxic to aquatic life. Because no direct measurements of ROS production in vivo were recorded in this study, we cannot exclude that the different mortality curves observed were, in part, due to differential sensitivity of respiratory inhibition. To substantiate our findings, we have also used paraquat to induce mitochondrial oxidative stress. Paraquat, upon its carrier-mediated uptake to the mitochondrial matrix, is reduced by NADPH dehydrogenases to form the paraquat radical cation that then reacts with oxygen to form superoxide (82), which is responsible for mitochondrial injury. Importantly, both long-lived species exhibited similar resistance to the two different mitochondrial stressors.

### *Conclusions*

To our knowledge, this is the first study comparing resistance to genotoxic stressors in bivalve mollusc species with disparate longevities. In line with previous studies of comparative stress resistance and longevity, our data extends, at least in part, the evidence for the hypothesis that an association exists between longevity and a general resistance to multiplex stressors, not solely oxidative stress. This work also provides justification for further investigation into the interspecies differences in stress response signatures induced by a diverse array of stressors in short-lived and long-lived bivalves, including pharmacological agents that elicit endoplasmic reticulum stress (24,31) and cellular stress caused by activation of innate immunity.

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