Longevity Is Linked to Mitochondrial Mutation Rates in Rockfish: A Test Using Poisson Regression

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Abstract

The mitochondrial theory of ageing proposes that the cumulative effect of biochemical damage in mitochondria causes mitochondrial mutations and plays a key role in ageing. Numerous studies have applied comparative approaches to test one of the predictions of the theory: That the rate of mitochondrial mutations is negatively correlated with longevity. Comparative studies face three challenges in detecting correlates of mutation rate: Covariation of mutation rates between species due to ancestry, covariation between life-history traits, and difficulty obtaining accurate estimates of mutation rate. We address these challenges using a novel Poisson regression method to examine the link between mutation rate and lifespan in rockfish (Sebastes). This method has better performance than traditional sister-species comparisons when sister species are too recently diverged to give reliable estimates of mutation rate. Rockfish are an ideal model system: They have long life spans with indeterminate growth and little evidence of senescence, which minimizes the confounding tradeoffs between lifespan and fecundity. We show that lifespan in rockfish is negatively correlated to rate of mitochondrial mutation, but not the rate of nuclear mutation. The life history of rockfish allows us to conclude that this relationship is unlikely to be driven by the tradeoffs between longevity and fecundity, or by the frequency of DNA replications in the germline. Instead, the relationship is compatible with the hypothesis that mutation rates are reduced by selection in long-lived taxa to reduce the chance of mitochondrial damage over its lifespan, consistent with the mitochondrial theory of ageing.

Key words: substitution rates, sister pairs, molecular evolution.

Introduction

The mitochondrial theory of ageing proposes that the cumulative effect of biochemical damage in mitochondria plays a key role in ageing. Mitochondria are the site of the oxidative phosphorylation pathway in which metabolism of molecules, such as carbohydrates and fats, releases electrons to generate ATP, which is then used by the cell as an energy source. This process produces reactive oxygen species (ROS) that contain free radicals that cause oxidative damage in cellular components, such as damaging DNA by oxidizing nucleotides (Barja 2002). Most of the ROS in a cell are produced in the mitochondria (Wallace et al. 2003), so the mitochondria are expected to sustain metabolism-induced damage. Mutations in the mitochondrial genome caused by ROS damage are thought to trigger a cascade of events: As mitochondria accumulate damage through metabolic activity, their ability to function and repair damage is degraded, so damage to biomolecules escalates, leading eventually to dysfunction or cell death (Gruber et al. 2007). One of the predictions of the mitochondrial theory of ageing is that rate of accumulation of mitochondrial mutations should be linked to the rate of cellular senescence (the cessation of cell division), which is closely interconnected with longevity (Tacutu et al. 2011). Accumulation of mitochondrial mutations has been linked to individual lifespan (Feng et al. 2001; Trifunovic et al. 2004; Yang et al. 2013), though not consistently (Speakman et al. 2004; Vermulst et al. 2007; Joyner-Matos et al. 2011). The causal links between metabolism, mitochondrial mutation, and individual ageing are a matter of debate (e.g., Martin et al. 1992; Partridge 2001; Jacobs 2003; Loeb et al. 2005).

Although most of the research in this field has focused on intraspecific associations between mitochondrial damage and individual life span, a complementary line of inquiry has investigated the differences between species. Some studies focus on understanding the biochemistry of particularly long-lived species, including turtles, naked mole rats, and clams (Holmes 2004; Buffenstein 2005; Philipp and Abele 2009). In other studies, predictions of the theory are compared with observations from species that differ in key characteristics, for example, comparing the relative amount of ROS produced by mammal species to similarly sized but longer-lived bird species (Skulachev 2004). A more formalized comparative approach is to use statistical analyses of data from many different species to ask whether patterns of longevity correlate with key aspects of the mitochondrial ageing theory, such as mutation rates, metabolism, or production of ROS (e.g., Barja 2002; Speakman 2005; Atanasov 2007; de Magalhaes et al. 2007; Nabholz et al. 2008).

Some comparative studies have included a selection of different species for which biochemical information can be...
obtained, typically from animals or cell lines used in laboratory experiments, such as rats, rabbits, cows, and humans (Kapahi et al. 1999; Barja and Herrero 2000; Foksinski et al. 2004). While providing an interesting starting point for investigation, taxonomically broad comparisons such as these are difficult to interpret in terms of causal mechanisms, because highly divergent taxa will differ in very many traits, not just those of interest to the study. Critically, studies that compare data from different species need to correct for the fact that species traits cannot be included as independent observations in a statistical analysis. Because species will tend to share many characteristics with their relatives due to common ancestry, known as the phylogenetic signal (Blomberg and Garland 2002; Revell et al. 2008), evolutionary relationships between species can create spurious patterns of association between traits (Felsenstein 1985; Lanfear et al. 2010; McGaughran and Holland 2010).

The nonindependence of species traits is further complicated by covariation between life-history traits. For example, in mammals and birds, body size typically scales with metabolism, lifespan, and rates of molecular evolution (Bromham 2009). Failure to account for this covariation can lead to false inference of causal relationships (Dowle et al. 2013; Glazier 2015). For example, although an association between basal metabolic rate and rate of molecular evolution has been observed (Martin and Palumbi 1993; Rand 1994; Santos 2012), studies that have accounted for covariation with other life-history variables have reported that basal metabolic rate has no significant explanatory power for variation in molecular substitution rates above and beyond its covariation with body size (Mooers and Harvey 1994; Bromham et al. 1996; Lanfear et al. 2007; Welch et al. 2008; Galtier, Jobson, et al. 2009).

One of the lines of evidence that has been used to support a link between mitochondrial damage and longevity is an association between rates of molecular evolution and maximum lifespan. Several studies have found that, in mammals and birds, mitochondrial synonymous substitution rates (which should reflect the mutation rate) are negatively correlated with lifespan, above and beyond covariation with other life-history parameters, such as body size and generation time (Nabholz et al. 2008; Welch et al. 2008). However, because these life history parameters in mammals scale together (Stearns 1983; Promislow and Harvey 1990; Charnov 1991), it is difficult to determine the mechanism underlying these patterns. In particular, two alternative (nonexclusive) explanations also fit the pattern of faster evolution of mitochondrial genome in mammals with shorter lives (Bromham 2009). Small, short-lived mammals might copy their genomes more per unit time, and therefore collect more DNA replication errors, a phenomenon often referred to as the generation time effect (e.g., Sarich and Wilson 1973; Ohta 1993; Bromham et al. 1996). Alternatively, large, long-lived mammals might invest more in DNA repair to avoid costly mutations because low fecundity magnifies the risk of deleterious mutations in the germline (e.g., Nabholz et al. 2008).

In addition to the problems of nonindependence due to phylogeny and covariation of life-history traits, analysis of the correlates of substitution rates presents special challenges. Because substitutions accumulate over time, substitution rates are essentially estimated from the count of substitutions. This means that estimates of substitution rate will be unreliable when the time elapsed is small compared with the substitution rate, so that the variance in estimates of substitution rates is larger between lineages that are more recently diverged, violating the assumption of homoscedasticity in a correlation analysis (Welch and Waxman 2008). One way to overcome the problem of unreliable rate estimates for recently diverged lineages is to exclude these species pairs from the analysis, by progressively removing pairs until there is no negative relationship between the square root of divergence time and the difference in the logarithm of substitution counts between sister species divided by the square root of divergence time, a test proposed by Welch and Waxman (2008). However, removing points that violate the Welch and Waxman (2008) test is not an ideal solution, because it reduces the size of the data set. Additionally, the Welch and Waxman test relies on estimates of divergence time. Some studies use divergence times estimated from molecular data, but this introduces a possible circularity. Ideally divergence time should be obtained from an independent estimate (e.g., fossil evidence), but this information is often not available for all pairs.

In this study, we address the challenges of phylogenetic nonindependence, covariation of species traits, and heteroscedasticity in estimates of substitution rate through a novel application of Poisson regression to investigate the relationship between mitochondrial mutation rate and longevity. Rather than including a selection of highly divergent species, we focus on variation in life history and substitution rate between 34 species in a single genus of fish, the rockfish (Sebastes), which includes some of the longest-lived fish species. Rockfish species vary in their maximum recorded lifespan from a decade to several centuries (Love et al. 2002). They have indeterminate growth (i.e., they continue to increase in body size throughout life). Fecundity increases with body size, as does larval recruitment (Finch 1994; Berkeley et al. 2004). There is no evidence of reproductive senescence in Sebastes: Oogenesis apparently continues into advanced age, with little evidence of the breakdown of oocytes which occurs in mammals (de Bruin et al. 2004). Rockfish therefore provide a useful contrast to mammals (Reznick et al. 2002), by breaking one of the confounding links between molecular evolution and life history: Because female gamete production continues throughout life and increases with age and size, fecundity is expected to scale positively with lifespan, as opposed to the negative relationship seen in mammals and many other taxa (fig. 1).

**New Approaches**

Although comparing species within a genus has the advantage that many of basic aspects of biology will be similar between species, it has the disadvantage that many sister species will be relatively recently diverged, and therefore not ideal for the estimation of substitution rates. Applying the Welch and Waxman (2008) test results in the exclusion of over 60% of data points in this study (fig. 2). We therefore...
develop a new method that makes it possible to overcome the problem, and use closely related taxa to look for correlates of rates of molecular evolution. This method models the accumulation of substitutions as a Poisson process, thus explicitly accounting for the heterogeneity of variance without having to exclude recently diverged lineages from the analysis. Another advantage of the method is that it does not require divergence time estimates.

To model the accumulation of substitutions, we make three assumptions. First, we assume that substitutions accumulate as a Poisson process; thus, the probability of observing \( S_1 \) substitutions in one species and \( S_2 \) substitutions in its sister species is:

\[
P(S_1, S_2) = e^{-T} \frac{\int_0^T \lambda_1(t) dt \int_0^T \lambda_2(t) dt}{S_1! S_2!},
\]

where \( \lambda_1(t) \) and \( \lambda_2(t) \) are the substitution rate of the two species in the pair, at time \( t \). \( T \) is the divergence time between the pair.

Second, we assume that species traits and substitution rates follow a log-log linear relationship. The log-log linear relationship between molecular evolution rate and species traits such as body size, metabolic rate, and generation time has been widely observed in empirical studies (e.g., Martin and Palumbi 1993; Gillooly et al. 2005). Using species 1 as an example, \( \lambda_1(t) = \lambda_1 e^{\beta x_1(t) - x_1} \), where \( x_1(t) \) is the trait value of species 1 at time \( t \), \( x_1 \) and \( x_2 \) are the substitution rate and trait value at the divergence time between the sister species, respectively, and \( \beta \) is the regression coefficient between the substitution rate and species trait on log-log scales.

**FIG. 1.** Illustration of the hypothesized relationships among species traits and mitochondrial mutation rate as suggested by previous studies. Signs in white circles indicate the directions of relationships. The mitochondrial theory of ageing is compatible with two observations: 1) A positive correlation between mitochondrial mutation rate and metabolic rate because metabolic rate may be positively linked to ROS production, and 2) a negative correlation (in bold) between mitochondrial mutation rate and longevity, which is the focus of this study. The two observations may connect to each other through environmental factors, such as temperature or depth for rockfish, so that fish living in deep water have a reduced metabolic rate and extended lifespan. However, the second observation may result indirectly from two other mechanisms that do not involve ROS: 1) If smaller species have shorter generation times, they may copy their genomes more per unit time and collect more DNA replication errors; longevity may therefore be negatively linked to mutation rate due to a tradeoff between fecundity and longevity. The life history of rockfish, indicated by signs in the gray circles, makes it possible to disentangle the direct link between mitochondrial mutation rate and longevity from these indirect links, because fecundity does not decrease with longevity as rockfish have indeterminate growth and do not show reproductive senescence.

**FIG. 2.** Plots of Welch and Waxman (2008) test for the rockfish data set and the simulated data sets. A random sample of the simulated data sets with high substitution rates and 30 species pairs is plotted to illustrate that the simulation can reproduce the heteroscedastic patterns in the rockfish mitochondrial synonymous substitution rates, as well as mitochondrial nonprotein-coding substitution rates and the rate of total substitutions across all mitochondrial sequences. A random sample of the simulated data sets with low substitution rates and 30 species pairs is plotted to show that the simulation can reproduce the heteroscedastic patterns in the rockfish mitochondrial and nuclear nonsynonymous substitution rates. A random sample of the simulated data sets with 500 species pairs under either high or low substitution rates is plotted to show the heteroscedastic patterns that may not be detected with 30 species pairs.
Third, because trait values of the sister species are fixed, we assume that species traits have evolved as a Brownian bridge, a Brownian motion process that satisfies conditions at both ends of the time interval. As the time integral of the exponential of a Brownian bridge has no closed form, we only use the expectation of trait value, for example, $$x_1(t) = X_1 + \frac{1}{2T}(X_2 - X_1),$$ where $$x_1 = \frac{x_1 + x_2}{2}. $$ The time integral of the expectation is $$\alpha \frac{2}{\beta} \left[1 - e^{\frac{\alpha}{\beta}(T - t)}\right].$$ $\alpha = 2\lambda T$ is a single parameter representing the expected genetic difference between sister species if substitution rate was not associated with species trait.

As a result, our model has $$N + 1$$ parameters for $$2N$$ species, including $\alpha$ for each species pair and the coefficient $\beta$. We used a maximum-likelihood approach to estimate those parameters, performed a likelihood ratio test on the effect of species traits on substitution rates, and approximated the standard error of the regression coefficient from the information matrix. Effect size can be calculated as pseudo-$R^2$ (Waldhør et al. 1998) for Poisson regression. As multiple traits and substitution rates were compared in the study, we adjusted the significance level for each test comparison by Bonferroni correction.

We compared the statistical power between our Poisson regression method and the traditional method of least-squares regression after the Welch and Waxman (2008) test by applying both methods to simulated data sets, in which substitution rates and species traits evolved by a correlated Brownian motion process. Each simulation had five parameters, including the average substitution rate, the rate of change in the substitution rate, the rate of evolution in the species trait value, the correlation coefficient between substitution rate and species trait, and the range of divergence times between sister species. Values of these parameters were chosen to reflect the range of values estimated from our rockfish data set. We describe how we estimated values of the five parameters in the Materials and Methods.

We used both our method and the least-squares regression after the Welch and Waxman (2008) test to analyze each of the simulated data sets using significance level 0.05. The null hypothesis is that there is no correlation between species trait values and substitution rate (i.e., $r = 0$). Thus, the proportion of data sets simulated under no correlation that show a significant result by a given method indicates the type I error of the method. The proportion of data sets simulated when trait values and substitution rate are correlated that successfully detect a significant relationship in the correct direction indicates the statistical power of the method, which is the chance that the method will correctly identify traits that are associated with substitution rates.

Results

For each species in 34 rockfish sister pairs, we gathered data on the maximum lifespan in years to reflect longevity, maximum number of eggs per breeding season ("clutch size" hereafter) to reflect fecundity, and total body length in centimeters to reflect body size. We also included the maximum depth in meters at which a species has been recorded, as depth has been shown to correlate with longevity in rockfish (Love et al. 2002). Values for some traits were not available for all species (supplementary table S1, Supplementary Material online). There are 34 species pairs for body length, 27 for maximum lifespan, 16 for clutch size, and 31 for maximum depth. To allow for different patterns of molecular evolution in different genomes and types of sequence, we constructed four different alignments: Mitochondrial protein-coding genes (cytochrome b [cytb], cytochrome oxidase c subunit 1 [COI]: 2,301 bp); mitochondrial nonprotein-coding sequences (12S, 16S, tRNAPro, tRNAThr, control: 1,486 bp); a nuclear protein-coding genes (recombination activating gene 2 [RAG2]: 711 bp); and a nuclear nonprotein-coding sequence (internal transcribed spacer 1 [ITS1]: 781 bp). For each sister pair, we estimated the number of substitutions for each of the four alignments, the total number of substitutions for an alignment of all mitochondrial sequences, and the total number of substitutions for an alignment of all nuclear sequences, using the baseml program of the PAML package (Yang 2007; see Materials and Methods; supplementary table S2, Supplementary Material online). In addition, we estimated the number of synonymous and nonsynonymous substitutions for each of the protein-coding alignments, using the codeml program of the PAML package (Yang 2007; supplementary table S2, Supplementary Material online).

Simulations Reproduce Patterns in Rockfish Substitutions

According to the Welch and Waxman test, sister pairs that are diverged for less than around 4 My show heteroscedasticity in estimates of mitochondrial synonymous substitution rate as well as rate of substitutions in mitochondrial nonprotein-coding alignment and rate of substitutions in the alignment of all mitochondrial sequences (fig. 2). The average rate of mitochondrial synonymous substitutions in the rockfish species analyzed here is about ten substitutions per alignment per million year and the rate changes at a rate estimated at two substitutions per million year according to our rockfish data set. We used these values to simulate data sets with "high substitution rate." These simulated data sets are able to reproduce the heteroscedastic pattern of substitution rates in the three mitochondrial alignments (fig. 2), so the statistical power estimated for these data sets can be used to suggest how reliable the results are for the three mitochondrial alignments.

The average rate of both mitochondrial and nuclear non-synonymous substitutions in the rockfish species analyzed here is about 0.2 substitutions per alignment per million year and the rate changes at a rate of 0.2 substitutions per million year. We used these values to simulate data sets with "low substitution rate." These simulated data sets are able to reproduce the heteroscedastic pattern in mitochondrial and nuclear nonsynonymous substitutions, where the Welch and Waxman test cannot confidently detect heteroscedasticity with the number of species pairs of our rockfish data set because many species have zero substitutions (fig. 2). Simulated data sets with more pairs suggest that all the
sister pairs show heteroscedasticity in estimates of the rate of substitutions that are as slow as the mitochondrial and nuclear nonsynonymous substitutions (fig. 2). Therefore, the statistical power estimated for data sets with low substitution rates can be used to suggest how reliable the results are for mitochondrial and nuclear nonsynonymous substitutions.

Nuclear synonymous substitution rate (~0.8 substitutions per alignment per million year), nuclear ITS substitution rate (~1 substitutions per alignment per million year), and rate of total substitutions across all nuclear sequences (~2 substitutions per alignment per million year) are in-between the rate of mitochondrial synonymous substitution and the rate of nonsynonymous substitutions. The statistical power to detect correlates of these substitution rates should therefore be within the range of the power for data sets with high substitution rate and data sets with low substitution rate.

Similarly, of the four species traits, body length evolves the most slowly at about 0.2 cm/My and clutch size evolves the fastest at about 0.9 eggs per million year, on logarithm scale. We used these two values to simulate data sets with “fast trait evolution” and “slow trait evolution.” The statistical power estimated for data sets with, for example, high substitution rate and fast trait evolution would reflect the ability of the methods to detect a link between a fast-evolving trait, such as clutch size, and a high substitution rate, such as the mitochondrial synonymous substitution, if such a link exists. The rate of evolution is about 0.4 years per million year for maximum lifespan and 0.6 m/My for maximum depth, so the statistical power to detect the influence of these traits on substitutions should be within the range of the power for data sets with fast trait evolution and data sets with slow trait evolution.

Power to Detect Correlates of Substitution Rates

Our Poisson regression method has similar type I error to the traditional method of applying least-squares regression to data diagnosed by the Welch and Waxman test, which fluctuates around the preset significance level 0.05 (fig. 3). Both methods have higher power with more species pairs, higher substitution rate, and faster evolving species traits (fig. 3). Our method makes two improvements. First, it increases the power to detect a link between a high substitution rate and a fast evolving trait with fewer than 30 species pairs (fig. 3A and B). It doubles the chance (~80%) to correctly detect a link between clutch size and mitochondrial synonymous, mitochondrial nonprotein coding, and total mitochondrial

![Figure 3](https://academic.oup.com/mbe/article-abstract/32/10/2633/1209051/1209051)
substitution rates, given that there are more than ten species pairs for clutch size in our rockfish data set (fig. 3A). Second, the power to detect a link between a low substitution rate and a fast evolving trait increases much faster with an increase in number of species pairs than the traditional method, which shows minimal increase in power with more species pairs sampled (fig. 3).

However, because all of the species traits except for clutch size have around 30 species pairs, the chances to detect links between species traits and mitochondrial and nuclear nonsynonymous substitution rates are all lower than 40% (fig. 3B). For traits evolving as slowly as body length, both methods have very low power (less than 30%) to detect the links with either low or high substitution rate, presumably because the traits are not variable enough to generate detectable variation in the count of substitutions. These power tests suggest that we should hesitate to interpret lack of a detectable relationship to indicate the absence of a link, either between species traits and nonsynonymous substitution rates or between body length and all types of substitution rates. However, we can be confident that when we do find a consistent relationship, it is indicative of a clear signal in the data.

Correlates of Substitution Rates in Rockfish
Correlations between pairs of species traits demonstrate that the three life-history traits (body length, maximum lifespan, and clutch size) are all strongly positively correlated (fig. 4). Body length and maximum lifespan increase with maximum depth, but clutch size does not (fig. 4). We predicted body length as a potential confounding factor for the link between other species traits and mutation rate (fig. 1), so we also derived residuals of these species traits from a regression against body length.

Synonymous substitution rate in mitochondrial protein-coding alignment is negatively correlated with maximum lifespan (table 1), regardless of whether body length is corrected for or not. Greater maximum lifespan is also significantly correlated with lower substitution rates in mitochondrial nonprotein-coding alignment and the alignment of all mitochondrial sequences (table 1 and supplementary fig. S1, Supplementary Material online). There is no correlation between maximum lifespan and nonsynonymous substitution rates. Clutch size is negatively correlated with substitution rate in the alignment of all mitochondrial sequences when body length is accounted for. Neither body length nor depth is associated with rates of molecular evolution in the mitochondria.

Body length, maximum lifespan, and clutch size are all negatively associated with substitution rate in the alignment of all nuclear sequences (table 1 and supplementary fig. S1, Supplementary Material online). Maximum lifespan is not correlated with nuclear substitution rates when body size is accounted for (table 1 and supplementary fig. S1, Supplementary Material online). Depth is not associated with rates of molecular evolution in any alignment of the two nuclear genes included in the study.

Discussion
Species-pair comparisons have been widely used to look for correlates of rates of molecular evolution (e.g., Lanfear et al. 2007; Welch et al. 2008; Galtier, Jobson, et al. 2009). Finding significant association between a species trait and variation in rates of molecular evolution provides a way of testing
hypotheses about the underlying causes of rates of molecular evolution. But estimates of rates of molecular evolution become less reliable for species that are more closely related (Welch and Waxman 2008). In this study, we have introduced a new method that provides a powerful way to use closely related species to look for correlated rates of molecular evolution. Compared with the commonly used approach of applying least-squares regression to data diagnosed by the Welch and Waxman test, the new method does not exclude data points with relatively unreliable rate estimates from the analysis and does not require divergence time estimates. The new method also has better statistical performance in terms of higher power to detect a link between rates of molecular evolution and a fast evolving trait.

Applying this Poisson regression method to rockfish, we find that longer-lived rockfish species tend to have lower mitochondrial synonymous substitution rates than their shorter-lived relatives, consistent with observations from mammals and birds (Nabholz et al. 2008; Welch et al. 2008; Galtier, Bier, et al. 2009). Synonymous mutations have very small selective effects, so difference in mitochondrial synonymous substitution rates between sister species should primarily reflect their difference in mitochondrial mutation rates (Kimura 1983). We also confirm that body size, longevity, and fecundity are all positively correlated in rockfish, unlike in mammals where fecundity tends to decrease with increasing size and longevity. Because rockfish show the opposite relationship between fecundity and longevity to mammals, it allows us to compare alternative explanations for the negative association between longevity and mitochondrial mutation rate. Here, we first discuss the implementation of our method, focusing on how to find the optimal sampling strategy to attain a particular level of power of our method. We then discuss three major explanations for the negative association between longevity and mitochondrial mutation rate: Evolutionary explanations, copy-frequency effect, and the metabolic rate hypothesis, in addition to the mitochondrial theory of aging.

Sampling Strategy to Look for Correlates of Rates of Molecular Evolution

We have applied simulations to estimate the power of our Poisson regression method to look for correlates of rates of molecular evolution. The method has higher power with more species pairs, higher substitution rate, and faster

Table 1. Likelihood Ratio Test, Effect Size, Mean, and Standard Error of the Association between Species Traits and Substitution Rates in Rockfish.

<table>
<thead>
<tr>
<th></th>
<th>Mitochondrial</th>
<th>Nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dS</td>
<td>dN</td>
</tr>
<tr>
<td><strong>Body length</strong></td>
<td>P</td>
<td>0.41</td>
</tr>
<tr>
<td>R²</td>
<td>0.005</td>
<td>0.008</td>
</tr>
<tr>
<td>( \beta )</td>
<td>0.1 ± 0.28</td>
<td>0.5 ± 0.21</td>
</tr>
<tr>
<td><strong>Maximum lifespan</strong></td>
<td>P</td>
<td>1.0</td>
</tr>
<tr>
<td>R²</td>
<td>0.168</td>
<td>0.097</td>
</tr>
<tr>
<td>( \beta )</td>
<td>-0.5 ± 0.24</td>
<td>-1.88 ± 1.80</td>
</tr>
<tr>
<td><strong>Clutch size</strong></td>
<td>P</td>
<td>0.04</td>
</tr>
<tr>
<td>R²</td>
<td>0.001</td>
<td>0.054</td>
</tr>
<tr>
<td>( \beta )</td>
<td>0.6 ± 0.11</td>
<td>1.0 ± 0.15</td>
</tr>
<tr>
<td><strong>Depth</strong></td>
<td>P</td>
<td>0.20</td>
</tr>
<tr>
<td>R²</td>
<td>0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>( \beta )</td>
<td>-0.2 ± 0.27</td>
<td>-1.3 ± 0.09</td>
</tr>
<tr>
<td><strong>Lifespan</strong></td>
<td>P</td>
<td>2.26</td>
</tr>
<tr>
<td>R²</td>
<td>0.246</td>
<td>0.097</td>
</tr>
<tr>
<td>( \beta )</td>
<td>-0.5 ± 0.23</td>
<td>-2.1 ± 1.27</td>
</tr>
<tr>
<td><strong>Residual depth</strong></td>
<td>P</td>
<td>1.0</td>
</tr>
<tr>
<td>R²</td>
<td>0.154</td>
<td>0.001</td>
</tr>
<tr>
<td>( \beta )</td>
<td>-0.3 ± 0.18</td>
<td>0.2 ± 1.44</td>
</tr>
<tr>
<td><strong>Depth</strong></td>
<td>P</td>
<td>3.88</td>
</tr>
<tr>
<td>R²</td>
<td>0.038</td>
<td>0.043</td>
</tr>
<tr>
<td>( \beta )</td>
<td>-0.2 ± 0.26</td>
<td>-2.4 ± 1.96</td>
</tr>
</tbody>
</table>

Note.—Species traits include body length, maximum lifespan, clutch size, and maximum depth. As maximum lifespan, clutch size, and maximum depth are all significantly correlated with body length, we also analyze the residuals from a regression of these traits against body length. Mitochondrial substitution rates include synonymous substitutions (dS), nonsynonymous substitutions (dN), and dN/dS in two mitochondrial protein-coding genes, substitutions in five mitochondrial nonprotein-coding sequences, and the total substitutions across these protein-coding genes and nonprotein-coding sequences. Nuclear substitution rates include synonymous substitutions (dS), nonsynonymous substitutions (dN), and dN/dS in nuclear protein-coding gene RAG2, substitutions in nuclear ITS sequence, and the total substitutions across the RAG2 and ITS sequences. Effect size is calculated by the pseudo-R² for Poisson regression. Likelihood ratio (D) value and the corresponding P value in italics indicate that the species trait is significantly correlated with the substitution rate, so that their regression coefficient (\( \beta \)) is significantly different from zero. Significance cutoff is 0.05 with Bonferroni correction.

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evolving species traits. The substitution rate is the number of substitutions occurred in a DNA alignment per unit time, so we can increase the substitution rate by sampling more and longer DNA sequences. Therefore, given a species trait, there are two ways to increase the power to detect a link between the species trait and rates of molecular evolution: Sampling more species pairs and sampling longer DNA alignment for each species. For example, to test for a correlation between mitochondrial nonsynonymous substitution rate and clutch size (fig. 3 under “fast” trait and “low” substitution), we would either need to sample 500 species pairs to attain a power over 80% (fig. 3D), or sample an alignment 50 times longer (ratio between high and low substitution rates) than the mitochondrial protein-coding alignment in the rockfish data set to make the mitochondrial nonsynonymous substitution rate comparable to that of a high substitution rate, and so attain 80% power with about ten species pairs (fig. 3A). Alternatively, we can sample both more specie pairs and more DNA data for each species. The optimal sampling strategy to attain a particular level of power can be found by simulating data sets under different combinations of number of species pairs and level of substitution rate and looking for the cheapest sampling strategy that gives the power or higher. We provide two R functions in the supplementary material, Supplementary Material online, one we used to simulate data set and the other to apply the Poisson regression method to both simulated data sets and empirical data set.

**Evolutionary Explanations for the Relationship between Longevity and Mitochondrial Mutation Rate**

It has been proposed that longer-lived species experience greater selection pressure to reduce mutation rates. Two evolutionary theories related to this idea are the disposable soma theory (Williams 1957; Kirkwood 1977) and the theory of mutation accumulation (Medawar 1952; Charlesworth 2000). The disposable soma theory argues that ageing arises from processes that have favorable effects in early life but deleterious effects in later life stages (Partridge and Barton 1993; Robins and Conneely 2014). In particular, early reproduction may jeopardize later survival by consuming resources and impairing somatic maintenance and repair mechanisms (Hamilton 1966; Partridge and Harvey 1985; Kirkwood 2005). However, in rockfish, there is no evidence of reproductive senescence. Longer-lived species tend to produce more eggs per season and older mothers produce higher quality larvae (Berkeley et al. 2004). As early reproduction in rockfish does not seem to jeopardize later survival, the disposable soma theory does not provide a good explanation of the negative association between maximum lifespan and rates of mitochondrial synonymous substitutions in rockfish.

The theory of mutation accumulation proposes that senescence is a consequence of an increasing mutation load in later stages of life (Medawar 1952; Partridge and Barton 1993; Robins and Conneely 2014). A long-lived organism will have a relatively higher mutation load because it has more time for deleterious mutations to occur. Furthermore, the rate of mutation itself may increase over an individual's lifespan if the DNA repair machinery declines in efficiency through the accumulation of deleterious mutations (Freitas and de Magalhães 2011). On a population level, deleterious mutations may be maintained in higher frequency in mutation-selection balance because selection against deleterious mutations becomes weaker when survival in the later stage of life matters comparatively less to lifetime reproductive success (Medawar 1952). As a result, the theory suggests that mutations have direct negative effects on longevity, so that to be a long-lived species requires a reduction in the mutation rate (Galtier, Jobson, et al. 2009). Mutation rate also determines the frequency of mutant alleles in mutation-selection balance when selection against deleterious mutations is weak (Partridge and Barton 1993).

The theory of mutation accumulation is compatible with the mitochondrial theory of ageing, which also predicts a stronger association between longevity and mitochondrial mutation rate than nuclear DNA because ROS are major causes of oxidative damage to both DNA sequences and DNA repair machinery (Barja and Herrero 2000; Barja 2002) and because most of the ROS in a cell are produced in the mitochondria (Wallace et al. 2003). Indeed, we find that maximum lifespan in rockfish is negatively associated with mitochondrial synonymous substitution rate but not nuclear synonymous substitution rate as estimated from RAG2 (nor with substitution rate in ITS). The association between maximum lifespan and rates of other types of nuclear substitutions also disappears when body length is accounted for. However, the small fraction of the nuclear genome we used and the low power of our method to detect correlates of nuclear substitution rates caution against interpreting the lack of a significant relationship between maximum lifespan and substitution rates in nuclear sequences as an indication that the nuclear and mitochondrial genomes are differently affected by selection associated with longevity.

**Copy-Frequency Effect**

The negative association between longevity and mitochondrial mutation rate could be due to the covariation between longevity and the frequency of DNA replication. For example, longer-lived mammals tend to have bigger bodies that take more cell generations to build, so it is expected that their germline cells will undergo fewer DNA replications per unit time, which might result in fewer DNA mutations given a rate of replication error (Bromham et al. 1996). In rockfish, longer-lived species tend to have bigger bodies, but they also produce more eggs per breeding season, and their reproductive capacities do not seem to decline with age, therefore longer-lived females may produce more eggs and undergo more germline DNA replications per unit time than their shorter-lived relatives. If replication frequency were a primary driver of differences in mutation rates between rockfish species, then we would expect a positive relationship between longevity and mutation rate, and between clutch size and mutation rate in rockfish (Bromham and Leys 2005). Yet mitochondrial synonymous substitution rate is lower in longer-lived rockfish
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Metabolic Rate Hypothesis

Metabolic rate measures the rate of energy production and the chief mechanism of energy production is the oxidative phosphorylation pathway, which produces ROS (Barja 2002). Higher metabolic rates are therefore hypothesized to correlate with higher rates of ROS production that might cause more mitochondrial mutations (Martin and Palumbi 1993; Galtier, Jobson, et al. 2009). Similar to mammals and birds, basal metabolic rate (often measured by resting oxygen consumption rate) is negatively related to body size in fish (Clarke and Johnston 1999; Seibel and Drazen 2007). Therefore metabolic rate might be negatively related to longevity in rockfish because maximum lifespan in rockfish is positively scaled with body length. In fish, the basal metabolic rate is positively linked to temperature (Clarke and Johnston 1999) and negatively linked to depth (Seibel and Drazen 2007). So the co-variation between metabolic rate and longevity in rockfish could also be mediated by environmental factors, as rockfish species living at greater depths tend to be longer-lived than those in shallower waters.

In fish, species assumed to have higher metabolic rates, for example, those found at lower latitudes or from shallower waters, were found to have higher rates of molecular evolution (Estabrook et al. 2007; Wright et al. 2011; Qiu et al. 2014). An observed association between temperature and rate of molecular evolution has been interpreted as the effect of temperature on metabolic rate carrying over to the influence of metabolic activity on mutation rate (e.g., Rohde 1992; Gillooly et al. 2005; Estabrook et al. 2007; Gillman and Wright 2014). However, these studies did not control for covariation in life history, and as a result may have been conflating the effects of metabolism and temperature with correlated patterns in other life-history traits, including longevity and fecundity. Studies on ectotherms that have controlled for covariation in life history have found no relationship between basal metabolic rate and rates of molecular evolution (Lanfear et al. 2007; Santos 2012). In rockfish, information on metabolic rate is scarce, but maximum depth is expected to be associated with variation in basal metabolic rate. Our results do not show a relationship between maximum depth and any type of mitochondrial substitution rate in rockfish. This result is unlikely to be due to the lack of power of our method, because maximum depth is the second fastest-evolving trait, so we expect to have sufficient power to detect a link between maximum depth and rate of molecular evolution.

There are several possible explanations for why maximum depth is not correlated with mitochondrial mutation rate in rockfish. We discuss two major explanations here. First, it is not clear to what extent the external temperature and mass specific metabolic rate would influence the germline in vertebrates (Lanfear et al. 2007). Female gametes are apparently held in a relatively quiescent state, with the minimum level of metabolic activity (de Paula et al. 2013). So variation in metabolic rates seems unlikely to play a strong role in determining mutation rates in female gamete mitochondria. Although mitochondria in sperm are active, they are almost always discarded from the embryo, and so do not contribute to the accumulation of mitochondrial substitutions (Allen 1995). Second, the association between ROS production and oxygen consumption is mediated by protein channels on the membrane of mitochondria, such as uncoupling proteins.
proteins (Speakman 2005). On one hand, the induction of uncoupling proteins decouples ROS production and oxygen consumption (Erlanson-Albertsson 2002), and it has been shown in fish to be associated with heat production under cold temperatures (Mark et al. 2006; Jastroch et al. 2007). On the other hand, the inactivation of uncoupling proteins increases the efficiency of mitochondria to produce ATP (Erlanson-Albertsson 2002). Oxygen deficiency and low food availability in the deep sea may require organisms to increase mitochondrial efficiency, producing more ROS for any given amount of oxygen consumed, whereas the total oxygen consumption is low. Either process could decouple the link between depth and total ROS production and therefore the link between maximum depth and mitochondrial synonymous substitution rate in rockfish.

Conclusion
We show that the rate of mitochondrial molecular evolution is negatively related to longevity in rockfish. The pattern of life-history variation in rockfish species allows us to conclude that this relationship is unlikely to be driven by selection to reduce mutation rate due to tradeoffs between longevity and fecundity, or by the frequency of DNA replications in the germline. This relationship is also unlikely to be due to the covariation between longevity and depth, because we found no evidence for a link between depth and mutation rate in rockfish. Instead, this relationship is compatible with the hypothesis that mutation rates are reduced by selection in long-lived taxa to reduce the chance of mitochondrial damage over the lifespan of an individual. This is the first study using organisms with negligible senescence to distinguish evidence for the mitochondrial theory of ageing from other various mechanisms. To assess the generality of these conclusions, we need to study other systems that show indeterminate growth and little evidence of senescence, such as sturgeon Acipenser, fish Allocyttus or Hoplostethus, tortoises Geochelone or Gopherus (Finch 1998), and turtles Emydidae or Terrapene (Miller 2001). For these genera, our study also provides a method to detect correlates of rates of molecular evolution using recently diverged species pairs that may not have accumulated enough DNA substitutions to reliably reflect their differences in rate of molecular evolution.

Materials and Methods
Data Collection
The genus Sebastes contains 110 species that are found at their highest diversity in the Northeast Pacific (Love et al. 2002). In order to compare rates of molecular evolution to differences in species traits, we selected pairs of closely related rockfish species, chosen to represent independent (nonoverlapping) sister pairs on the phylogeny (Harvey and Purvis 1991; supplementary fig. S1, Supplementary Material online). Each lineage in the selected species pair has had the same amount of time to accumulate substitutions since they split from their shared common ancestor, and thus any differences in their branch lengths should reflect a difference in their net substitution rates (Lanfear et al. 2010). We used a published phylogeny of ray-finned fishes (Rabosky et al. 2013), from which we extracted the Sebastes clade to choose pairs of closely related rockfish species, plus an outgroup. The Rabosky et al. (2013) phylogeny, which includes 7,822 fish species, is based on 13 sequences (12S, 16S, 4C4, cytB, ENC1, gyt, myh6, plagl2, rag1, rhodopsin, sreb2, tbr1, zic1), only two of which overlap with our data set. Pairs were chosen to maximize the number of possible comparisons with data for the four species traits (lifespan, clutch size, body length, and depth).

We gathered data on longevity (maximum life span in years), fecundity (maximum number of eggs per breeding season), total body length (cm), and the maximum water depth where a species was recorded (m) from Fishbase (www.fishbase.org, last accessed October 2013) and published sources (Love et al. 2002). Not all traits are available for all species (supplementary material, Supplementary Material online). We selected 34 sister pairs for which body size data were available, and subsets of these comparisons also had data for longevity (n = 27), fecundity (n = 16), and maximum depth (n = 31). All trait values were log-transformed to approach normality.

We estimated rates of molecular evolution for seven mitochondrial sequences: cytB, COI, 12S rRNA, 16S rRNA, tRNA proline, tRNA threonine, and control region (Hyde and Vetter 2007). For comparison, we also included two available nuclear sequences for these species: RAG2 and ITS1. Sequence alignments were obtained from TreeBase (ID S2025). Protein-coding exons were aligned in frame using amino acid translation as a guide, with the MUSCLE translation plugin (Edgar 2004). To allow for different patterns of molecular evolution in different genomes or types of sequence, we constructed four different alignments: Mitochondrial protein-coding genes (cytB, COI; 2,301 bp in total), mitochondrial nonprotein-coding sequences (12S, 16S, tRNA Pro, tRNA Thr, control; 1,486 bp in total), nuclear protein-coding genes (RAG2; 711 bp), and nuclear ITS (781 bp). All sequence alignments are deposited at http://figshare.com/.

For each sister pair, we estimated branch length (the average number of substitutions per site) for each of the four alignments using maximum likelihood and a GTR + G model suggested by Hyde and Vetter (2007) in the baseml program of the PAML package (Yang 2007). For mitochondrial protein-coding genes (cytB, COI) and the nuclear protein-coding gene (RAG2), we also estimated synonymous and nonsynonymous branch lengths using the GY94 codon substitution model suggested by Goldman and Yang (1994) in the codeml program of the PAML package (Yang 2007), with dN/dS values free to vary across the tree. The count of substitutions is the branch length multiplied by the length of the alignment.

Power Estimation
To estimate the statistical power of our method and the traditional method of least-squares regression after the Welch and Waxman test, we simulated 100 data sets under each of the combinations of four conditions. The combinations of four conditions are: 1) The number of sister pairs: 10,
30, 100, or 500 pairs; 2) the substitution rate: Fast substitution (average substitution rate = $e^{-2}$ substitutions per sequence per time step and rate of evolution = $10^{-3}$ per time step) versus slow substitution (average substitution rate = $e^{-6}$ substitutions per sequence per time step and rate of evolution = $10^{-4}$ per time step); 3) the rate of evolution of the species trait: Fast trait (rate of evolution = 0.1 per time step) versus slow trait (rate of evolution = 0.01 per time step); 4) the correlation coefficient between substitution rate and species trait: $r = 1, 0.5, 0, -0.5$, or $-1$. A time step in the simulation is 0.01 My. The simulation duration for each sister pair was drawn at random from a uniform distribution between 0 and 1,000 time steps, that is, 10 My, the maximum divergence time between rockfish sister species estimated by Rabosky et al. (2013).

Values of these conditions are chosen to reflect the range of values estimated from our rockfish data set. We estimate the average substitution rate as the average of substitution rates over all species included in the data set, where the substitution rate of a species is calculated using the number of substitutions in the species since divergence from its sister species divided by their divergence time as estimated by Rabosky et al. (2013). We estimate the rate of evolution of a trait as the square root of the average of trait variance between a rockfish sister species divided by twice the divergence time of that species pair. To estimate the rate of evolution of substitution rate $\delta$, we first assume that substitution rate evolves through a Brownian motion process with rate $\alpha$, so that the substitution rate is a random variable changing over time. We then estimate the number of substitutions in a species by integrating the substitution rate over the divergence time $T$ of the species, which is equivalent to the time integral of the Brownian motion. Thus the expected variance of the number of substitutions between a species pair is twice the variance of the integrated Brownian motion, which is $\delta^2 T^3 / 3$. Finally, we can calculate $\delta$ from the variance of the number of substitutions between a species pair multiplied by $3/2 T^3$, averaging over all the species pairs in the rockfish data set.

**Supplementary Material**

Supplementary material, figure S1, and tables S1 and S2 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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**References**


