MBE Advance Access published June 5, 2015

Submitted as Article in the Discoveries section

Title: Longevity is linked to mitochondrial mutation rates in rockfish: a test using Poisson regression

Authors: Xia Hua[1*], Peter Cowman[1,2], Dan Warren[1,3], Lindell Bromham[1]

Affiliations:

[1] Centre for Macroevolution and Macroecology, Research School of Biology, Australian National University, Canberra ACT 0200 Australia.

[2]Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06511, USA

[3]Department of Biology, Macquarie University, North Ryde, NSW 2109, Australia

[*]Correspondence to: xia.hua@anu.edu.au

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.

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).

While most of the research in this field has focussed 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 to 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 formalised 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; Fokskinki 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 non-independence 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 2014). For example, while an association between 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 et al. 2009b).

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 (non-exclusive) 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 non-independence 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 to 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 dataset. 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 non-independence, 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 (Figure 1).

New Approaches

While 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 the present study (Figure 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:

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

where $\lambda_1(t)$, $\lambda_2(t)$ 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, $\lambda_1(t) = \lambda_c e^{\beta[x_1(t)-x_c]}$, where $x_1(t)$ is the trait value of species 1 at time *t*, λ_c and x_c are the substitution rate and trait value at the divergence time between the sister species, respectively, and β is the regression coefficient between the substitution rate and species trait on log-log scales.

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. Since the time integral of the exponential of a Brownian bridge has no closed form, we only use the expectation of trait value, for example, $x_1(t) = X_1 + \frac{t}{2T}(X_2 - X_1)$, $x_c = \frac{X_1+X_2}{2}$. The time integral of the expectation is $\frac{\alpha}{\beta(X_2-X_1)}[1 - e^{\frac{\beta(X_1-X_2)}{2}}]$. $\alpha = 2\lambda_c T$ 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 2*N* species, including α for each species pair and the coefficient β. 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 (Waldhör et al. 1998) for Poisson regression. Since 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 datasets, 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 dataset. 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 analyse each of the simulated datasets 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 datasets simulated under no correlation that show a significant result by a given method indicates the type I error of the method. The proportion of datasets 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 centimetres to reflect body size. We also included the maximum depth in metres 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 (Table S1). 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 (*cytb, COI*: 2301 bp); mitochondrial non-protein-coding sequences (*12S*, *16S*, *tRNAPro*, *tRNAThr*, *control*: 1486 bp); a nuclear protein-coding genes (*RAG2*: 711 bp); and a nuclear non-protein coding sequence (*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; Table S2). 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; Table S2).

Simulations reproduce patterns in rockfish substitutions

According to the Welch and Waxman test, sister pairs that are diverged for less than around 4 million years (myr) show heteroscedasticity in estimates of mitochondrial synonymous substitution rate as well as rate of substitutions in mitochondrial non-protein-coding alignment and rate of substitutions in the alignment of all mitochondrial sequences (Figure 2). The average rate of mitochondrial synonymous substitutions in the rockfish species analysed here is about 10 substitutions per alignment per myr and the rate changes at a rate estimated at 2 substitutions per myr according to our rockfish dataset. We used these values to simulate datasets with 'high substitution rate'. These simulated datasets are able to reproduce the heteroscedastic pattern of substitution rates in the three mitochondrial alignments (Figure 2), so the statistical power estimated for these datasets can be used to suggest how reliable the results are for the three mitochondrial alignments.

The average rate of both mitochondrial and nuclear nonsynonymous substitutions in the rockfish species analysed here is about 0.2 substitutions per alignment per myr and the rate changes at a rate of 0.2 substitutions per myr. We used these values to simulate datasets with 'low substitution rate'. These simulated datasets 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 dataset because many species have zero substitutions (Figure 2). Simulated datasets 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 nonsysnonymous substitutions (Figure 2). Therefore, the statistical power estimated for datasets 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 myr), nuclear ITS substitution rate (~1 substitutions per alignment per myr), and rate of total substitutions across all nuclear sequences (~2 substitutions per alignment per myr) 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 datasets with high substitution rate and datasets with low substitution rate.

Similarly, of the four species traits, body length evolves the most slowly at about 0.2 centimetres per myr and clutch size evolves the fastest at about 0.9 eggs per myr, on logarithm scale. We used these two values to simulate datasets with 'fast trait evolution' and 'slow trait evolution'. The statistical power estimated for datasets 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 myr for maximum lifespan and 0.6 metres per myr 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 datasets with fast trait evolution and datasets 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 (Figure 3). Both methods have higher power with more species pairs, higher substitution rate, and faster evolving species traits (Figure 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 (Figure 3A, 3B). It doubles the chance (~ 80%) to correctly detect a link between clutch size and mitochondrial synonymous, mitochondrial non-protein coding, and total mitochondrial substitution rates, given that there are more than 10 specie pairs for clutch size in our rockfish dataset (Figure 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 (Figure 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% (Figure 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 (Figure 4). Body length and maximum lifespan increase with maximum depth, but clutch size does not (Figure 4). We predicted body length as a potential confounding factor for the link between other species traits and mutation rate (Figure 1), so we also derived residuals of these species traits from a regression against body length.

Synonymous substitution rate in mitochondrial protein-coding alignment are negatively correlated with maximum life span (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 non-protein coding alignment and the alignment of all mitochondrial sequences (Table 1, Figure S1). 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 are 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, Figure S1). Maximum lifespan is not correlated with nuclear substitution rates when body size is accounted for (Table 1, Figure S1). 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 et al. 2009b). 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 of rates of molecular evolution. Compared to 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 et al. 2009a). 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 ageing.

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 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 (Figure 3 under 'fast' trait and 'low' substitution), we would either need to sample 500 species pairs to attain a power over 80% (Figure 3D), or sample an alignment 50 times longer (ratio between high and low substitution rates) than the mitochondrial protein-coding alignment in the rockfish dataset to make the mitochondrial nonsynonymous substitution rate comparable to that of a 'high' substitution rate, and so attain 80% power with about 10 species pairs (Figure 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 datasets 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 materials, one we used to simulate dataset and the other to apply the Poisson regression method to both simulated datasets and empirical dataset.

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 favourable 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; Patridge 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). Since 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 et al. 2009b). 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 disappear 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 takes 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 species, and neither mitochondrial nor nuclear synonymous substitution rates are associated with clutch size. These results are unlikely to be due to the statistical errors of our method, because the estimated type I error is around 0.05 and the power is up to 80% for the mitochondrial results and the regression coefficients are consistently negative.

The copy-frequency effect is a good explanation for many observed patterns in rate of molecular evolution, such as the widespread observation of a generation time effect (e.g. Mooers and Harvey 1994; Bromham et al. 1996; Smith and Donaghue 2008; Thomas et al. 2010); higher rates of molecular evolution in highly eusocial hymenopterans (Bromham and Leys 2005); faster rates of molecular evolution in shorter plants (Lanfear et al. 2013a; Bromham et al. 2015); and faster mutations in sequences that are carried more often in male germline than in female germline (Ellegren and Fridolfsson 1997; Whittle and Johnston 2002). So why do we not find evidence that the copy-frequency effect is a primary driver of differences in mutation rates between rockfish species?

Population size could be one of the possible reasons. In mammals, large, longer-lived species with low fecundity also tend to have smaller effective population sizes, and reductions in effective population size are expected to increase the fixation rate of nearly neutral mutations, resulting in more substitutions (Ohta 1992; Charlesworth 2009; Woolfit 2009; Lanfear et al. 2013b). In rockfish, we expect the opposite pattern because longer-lived rockfish tend to have higher fecundity without a reduction in offspring quality, so longer-lived species with larger clutch sizes may have larger effective population sizes (Caballero 1994). Under large effective population size, selection is comparatively more efficient at removing slightly deleterious mutations, thereby resulting in fewer nonsynonymous substitutions (Ohta 1992; Charlesworth 2009; Woolfit 2009). This prediction is supported by the negative links between clutch size and nuclear ITS substitution rate and the total substitution rate across all mitochondrial and nuclear sequences, because species with larger

clutch sizes may have larger effective population sizes. Population size may also have a small effect on synonymous substitution rate in rockfish because although selective effects on synonymous substitutions are small (of the order of 1×10^{-6}; Chamary et al. 2006), rockfish clutch size is large (of the order of 1×10^{6}; Table S1). The fixation rate of a nearly neutral mutations is largely determined by the product of effective population size and selective effects, with a larger product predicting faster loss of mutations and thus fewer substitutions (Ohta 1992). This effect may counteract the copy-frequency effect and therefore might explain the lack of signals of copy-frequency effect on differences in mutation rates between rockfish species.

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 et al. 2009b). 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 covariation 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. While 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 (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, while 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 allow 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 *Emydoidea* 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 (non-overlapping) sister pairs on the phylogeny (Harvey and Purvis 1991, Figure S1). 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*, *glyt*, *myh6*, *plagl2*, *rag1*, *rhodopsin*, *sreb2*, *tbr1*, *zic1*), only two of which overlap with our dataset. 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) and published sources (Love et al. 2002). Not all traits are available for all species (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: *cytochrome b* (*cytb*), *cytochrome oxidase c subunit 1* (*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: *recombination activating gene 2* (*RAG2*) and *internal transcribed spacer 1* (*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*; 2301 bp in total), mitochondrial non-protein coding sequences (*12S, 16S, tRNAPro, tRNAThr, control*; 1486 bp in total), nuclear protein-coding genes (*RAG2*; 711 bp) and nuclear *ITS* (781 bp).

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 datasets 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 myr. The simulation duration for each sister pair was drawn at random from a uniform distribution between 0 and 1000 time steps, i.e. 10 myr, 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 dataset. We estimate the average substitution rate as the average of substitution rates over all species included in the dataset, 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 δ, we first assume that substitution rate evolves via a Brownian motion process with rate δ, 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 δ from the variance of the number of substitutions between a species pair multiplied by $3/2T^3$, averaging over all the species pairs in the rockfish dataset.

References

Allen J. 1995. Separate sexes and the mitochondrial theory of ageing. *J theor Biol*. 180:135–140.

Atanasov AT. 2007. The linear allometric relationship between total metabolic energy per life span and body mass of mammals. *Biosystems* 90:224–233.

Barja G. 2002. Rate of generation of oxidative stress-related damage and animal longevity. *Free Radical Bio Med.* 33:1167–1172.

Barja G, Herrero A. 2000. Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. *FASEB J.* 14:312.

Bartosch-Harlid A, Berlin S, Smith NGC, ller AP, Ellegren H. 2003. Life history and the male mutation bias. *Evolution* 57:2398–2406.

Berkeley SA, Chapman C, Sogard SM. 2004. Maternal age as a determinant of larval growth and survival in a marine fish, *Sebastes melanops*. *Ecology* 85:1258–1264.

Blomberg SP, Garland T Jr. 2002. Tempo and mode in evolution: phylogenetic inertia, adaptation and comparative methods. *J. Evol. Biol.* 15:899–910.

Bromham L. 2009. Why do species vary in their rate of molecular evolution? *Biol Lett.* 5:401–404.

Bromham L, Hua X, Lanfear R, Cowman PF. 2015. Exploring the relationships between mutation rates, life history, genome size, environment and species richness in flowering plants. *Am Nat.* 185:507–524.

Bromham L, Leys R. 2005. Sociality and rate of molecular evolution. *Mol Biol Evol*. 22:1393–1402.

Bromham L, Rambaut A, Harvey PH. 1996. Determinants of rate variation in mammalian DNA sequence evolution. *J Mol Evol.* 43:610–621.

Buffenstein R. 2005. The naked mole-rat: a new long-living model for human ageing research. *J Gerontol A Biol Sci Med Sci.* 60:1369–1377.

Caballero A. 1994. Developments in the prediction of effective population size. *Heredity* 73:657–679.

Chamary JV, Parmley JL, Hurst LD. 2006. Hearing silence: Non-neutral evolution at synonymous sites in mammals. *Nat Rev Genet*. 7:98–108.

Charlesworth B. 2000. Fisher, Medawar, Hamilton and the evolution of ageing. *Genetics* 156:927–931.

Charlesworth B. 2009. Effective population size and patterns of molecular evolution and variation. *Nature Rev Genet.* 10:195–205.

Charnov EL. 1991. Evolution of life history variation among female mammals. *Proc Natl Acad Sci USA*. 88:1134–1137.

Clarke A, Johnston NM. 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. *J Anim Ecol*. 68:893–905.

de Bruin J–P, Gosden RG, Finch CE, Leaman BM. 2004. Ovarian ageing in two species of long-lived rockfish, *Sebastes aleutianus* and *S. alutus*. *Biol Reprod.* 71:1036–1042.

de Magalhaes JP, Costa J, Church GM. 2007. An analysis of the relationship between metabolism, developmental schedules, and longevity using phylogenetic independent contrasts. *J Gerontol A Biol Sci Med Sci*. 62:149–160.

de Paula WBM, Lucas CH, Agip A-NA, Vizcay-Barrena G, Allen JF. 2013. Energy, ageing, fidelity and sex: oocyte mitochondrial DNA as a protected genetic template. *Phil Trans R Soc B.* 368: 20120267.

Dowle E, Morgan-Richards M, Trewick S. 2013. Molecular evolution and the latitudinal biodiversity gradient. *Heredity* 110:501–510.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792–97.

Ellegren H, Fridolfsson AK. 1997. Male-driven evolution of DNA sequences in birds. *Nat Genet.* 17:182–184.

Erlanson-Albertsson C. 2002. Uncoupling proteins – a new family of proteins with unknown function. *Nutr Neurosci*. 5:1–11.

Estabrook G, Smith G, Dowling T. 2007. Body mass and temperature influence rates of mitochondrial DNA evolution in North American cyprinid fish. *Evolution* 61:1176–1187.

Felsenstein J. 1985. Phylogenies and the comparative method. *Am Nat*.125:1–15.

Feng J, Bussière F, Hekimi S. 2001. Mitochondrial electron transport is a key determinant of life span in *Caenorhabditis elegans*. *Dev Cell.* 1:633–644.

Finch CE. 1994. Longevity, senescence, and the genome. Chicago: University of Chicago Press.

Finch CE. 1998. Variations in senescence and longevity include the possiblity of negligible senescence. *J Gerontol A Biol Sci Med Sci.* 53:B235–B239.

Fokskinki M, Rozalski R, Guz J, Ruszkowska B, Sztukowska P, Piwowarski M, Klungland A, Olinski R. 2004. Urinary exrection of DNA repair products correlates with metabolic rates as well as with maximum life spans of different mammalian species. *Free Radical Bio Med.* 37:1449–1454.

Freitas AA, de Magalhães JP. 2011. A review and appraisal of the DNA damage theory of ageing. *Mutat Res.* 728:12–22.

Galtier N, Blier PU, Nabholz B. 2009. Inverse relationship between longevity and evolutionary rate of mitochondrial proteins in mammals and birds. *Mitochondrion* 9:51–57.

Galtier N, Jobson RW, Nabholz B, Glemin S, Blier PU. 2009. Mitochondrial whims: metabolic rate, longevity and the rate of molecular evolution. *Biol Lett*. 5:413–416.

Gillman LN, Wright SD. 2014. Species richness and evolutionary speed: the influence of temperature, water and area. *J Biogeogr*. 41:39–51.

Gillooly JF, Allen AP, West GB, Brown JH. 2005. The rate of DNA evolution: Effects of body size and temperature on the molecular clock. *Proc Natl Acad Sci USA*. 102:140–145.

Glazier DS. 2014. Is metabolic rate a universal 'pacemaker'for biological processes? *Biol Rev*. DOI: 10.1111/brv.12115.

Goetting-Minesky MP, Makova KD. 2005. Mammalian male mutation bias: impacts of generation time and regional variation in substitution rates. *J Mol Evol*. 63:537–544.

Goldman N, Yang Z. 1994. A codon-based model of nucleotide substitution for protein-coding DNA sequences. *Mol Biol Evol.* 11:725–736.

Gruber J, Schaffer S, Halliwell B. 2007. The mitochondrial free radical theory of ageing--where do we stand? *Front Biosci.* 13:6554–6579.

Hamilton WD. 1966. The moulding of senescence by natural selection. *J Theor Biol.* 12:12–45.

Harvey PH, Purvis A. 1991. Comparative methods for explaining adaptations. *Nature* 351:619–624.

Holmes DJ. 2004. Naturally long-lived animal models for the study of slow ageing and longevity. *Ann N Y Acad Sci.* 1019:483–485.

Hyde JR, Vetter RD. 2007. The origin, evolution, and diversification of rockfishes of the genus *Sebastes* (Cuvier). *Mol Phylogenet Evol*. 44:790–811.

Jacobs HT. 2003. The mitochondrial theory of ageing: dead or alive? *Ageing Cell* 2:11–17.

Jastroch M, Buckingham JA, Helwig M, Klingenspor M, Brand MD. 2007. Functional characterisation of UCP1 in the common carp: uncoupling activity in liver mitochondria and cold-induced expression in the brain. *J Comp Physiol B.* 177:743–752.

Joyner-Matos J, Bean LC, Richardson HL, Sammeli T, Baer CF. 2011. No evidence of elevated germline mutation accumulation under oxidative stress in *Caenorhabditis elegans*. *Genetics* 189:1439–1447.

Kapahi P, Boulton ME, Kirkwood TB. 1999. Positive correlation between mammalian life span and cellular resistance to stress. *Free Radical Biol Med.* 26:495–500.

Kimura M. 1983. The Neutral Theory of Molecular Evolution. Cambridge: Cambridge University Press.

Kirkwood TB. 1977. Evolution of ageing. *Nature* 270:301–304.

Kirkwood TB. 2005. Understanding the odd science of ageing. *Cell* 120:437–447.

Lanfear R, Ho SYW, Davies TJ, Moles ATA, L., Swenson NG, Warman L, Zanne AE, Allen AP. 2013. Taller plants have lower rates of molecular evolution: the rate of mitosis hypothesis. *Nat Commun.* 4:1879.

Lanfear R, Kokko H, Eyre-Walker A. 2013. Population size and the rate of evolution. *Trends Ecol Evol.* 29:33–41.

Lanfear R, Thomas JA, Welch JJ, Bromham L. 2007. Metabolic rate does not calibrate the molecular clock. *Proc Natl Acad Sci USA*. 104:15388–15393.

Lanfear R, Welch JJ, Bromham L. 2010. Watching the clock: Studying variation in rates of molecular evolution. *Trends Ecol Evol.* 25:495–503.

Li WH, Yi S, Makova KD. 2002. Male-driven evolution. *Curr Op Genet Dev*. 12:650–656.

Loeb LA, Wallace DC, Martin GM. 2005. The mitochondrial theory of ageing and its relationship to reactive oxygen species damage and somatic mtDNA mutations. *Proc Natl Acad Sci USA*. 102:18769–18770.

Love MS, Yoklavich M, Thorsteinson LK. 2002. The rockfishes of the northeast Pacific. Berkeley: University of California Press.

Mark FC, Lucassen M, Pörtner HO. 2006. Thermal sensitivity of uncoupling protein expression in polar and temperate fish. *Comp Biochem Physiol Part D Genomics Proteomics.* 1:365–374.

Martin AP, Naylor G, Palumbi SR. 1992. Rates of mitochondrial DNA evolution in sharks are slow compared with mammals. *Nature* 357:153–155.

Martin AP, Palumbi SR. 1993. Body size, metabolic rate, generation time and the molecular clock. *Proc Natl Acad Sci USA*. 90:4087–4091.

McGaughran A, Holland BR. 2010. Testing the effect of metabolic rate on DNA variability at the intra-specific level. *PLoS ONE*. 5:e9686.

Medawar PB. 1952. An unsolved problem of biology. London: HK Lewis.

Miller JK. 2001. Escaping senescence: demographic data from the three-toed box turtle (*Terrapene carolina triunguis*). *Exp Gerontol*. 36:829–832

Mooers AO, Harvey PH. 1994. Metabolic rate, generation time and the rate of molecular evolution in birds. *Mol Phylogenet Evol*. 3:344–350.

Nabholz B, Glemin S, Galtier N. 2008. Strong variations of mitochondrial mutation rate across mammals - the longevity hypothesis. *Mol Biol Evol*. 25:120–130.

Ohta T. 1992. The nearly neutral theory of molecular evolution. *Ann Rev Ecol Syst.* 23:263–286.

Ohta T. 1993. An examination of the generation time effect on molecular evolution. *Proc Natl Acad Sci USA*. 90:10676–10680.

Partridge L. 2001. Evolutionary theories of ageing applied to long-lived organisms. *Exp Gerontol.* 36:641–650.

Patridge L, Barton NH. 1993. Optimality, mutation and the evolution of ageing. *Nature* 362:305–311.

Patridge L, Harvey PH. 1985. Costs of reproduction. *Nature* 316:20.

Philipp EE, Abele D. 2009. Masters of longevity: lessons from long-lived bivalves–a mini-review. *Gerontology* 56:55–65.

Promislow DEL, Harvey PH. 1990. Living fast and dying young: A comparative analysis of life-history variation among mammals. *J Zool.* 220:417–437.

Qiu F, Kitchen A, Burleigh JG, Miyamoto MM. 2014. Scombroid fishes provide novel insights into the trait/rate associations of molecular evolution. *J Mol Evol.* 78:338–348.

Rabosky DL, Santini F, Eastman J, Smith SA, Sidlauskas B, Chang J, Alfaro ME. 2013. Rates of speciation and morphological evolution are correlated across the largest vertebrate radiation. *Nat Commun.* 4:1958.

Rand DM. 1994. Thermal habit, metabolic rate and the evolution of mitochondrial DNA. *Trends Ecol Evol.* 9:125–131.

Revell LJ, Harmon LJ, Collar DC. 2008. Phylogenetic signal, evolutionary process, and rate. *Syst. Biol.* 57:591–601.

Reznick D, Ghalambor C, Nunney L. 2002. The evolution of senescence in fish. *Mech Ageing Dev*. 123:773–789.

Robins C, Conneely KN. 2014. Testing evolutionary models of senescence: traditional approaches and future directions. *Hum Genet.* 133:1451–1465.

Rohde K. 1992. Latitudinal gradients in species diversity: the search for the primary cause. *Oikos* 65:514–527.

Santos JC. 2012. Fast molecular evolution associated with high active metabolic rates in poison frogs. *Mol Biol Evol.* 29:2001–2018.

Sarich VM, Wilson AC. 1973. Generation time and genomic evolution in primates. *Science* 179:1144–1147.

Seibel BA, Drazen JC. 2007. The rate of metabolism in marine animals: environemtnal constraints, ecological demands and energetic opportuniries. *Phil Trans R Soc B*. 362:2061–2078.

Skulachev VP. 2004. Mitochondria, reactive oxygen species and longevity: some lessons from the Barja group. *Ageing Cell* 3:17–19.

Smith SA, Donoghue MJ. 2008. Rates of molecular evolution are linked to life history in flowering plants. *Science* 322:86–89.

Speakman JR. 2005. Body size, energy metabolism and lifespan. *J exp Biol*. 208:1717–1730.

Speakman JR, Talbot DA, Selman C, Snart S, McLaren JS, Redman P, Krol E, Jackson DM, Johnson MS, Brand MD. 2004. Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Ageing Cell* 3:87–95.

Stearns SC. 1983. The influence of size and phylogeny on patterns of covariation among life-history traits in the mammals. *Oikos* 41:173–187.

Tacutu R, Budovsky A, Yanai H, Fraifeld VE. 2011. Molecular links between cellular senescence, longevity and age-related diseases - a systems biology perspective. *Aging* 3:1178–1191.

Thomas JA, Welch JJ, Lanfear R, Bromham L. 2010. A generation time effect on the rate of molecular evolution in invertebrates. *Mol Biol Evol.* 27:1173–1180.

Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohooly YM, Gidlof S, Oldfors A, Wibom R, et al. 2004. Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* 429:417–423.

Vermulst M, Bielas JH, Kujoth GC, Ladiges WC, Rabinovitch PS, Prolla TA, Loeb LA. 2007. Mitochondrial point mutations do not limit the natural lifespan of mice. *Nat Genet*. 39:540–543.

Waldhör T, Haidinger G, Schober E. 1998. Comparison of R^2 measures for Poisson regression by simulation. *J Epidemiol Biostatist.* 3:209–215.

Wallace D, Ruiz-Pesini E, Mishmar D. 2003. mtDNA variation, climatic adaptation, degenerative diseases, and longevity. In: Cold Spring Harbor symposia on quantitative biology. Cold Spring Harbor: Cold Spring Harbor Laboratory Press. p. 471–478.

Welch JJ, Bininda-Emonds ORP, Bromham L. 2008. Correlates of substitution rate variation in mammalian protein-coding sequences. *BMC Evol Biol*. 8:53.

Welch JJ, Waxman D. 2008. Calculating independent contrasts for the comparative study of substitution rates. *J theor Biol.* 251:667–678.

Whittle C-A, Johnston MO. 2002. Male-driven evolution of mitochondrial and chloroplastidial DNA sequences in plants. *Mol Biol Evol*. 19:938–949.

Williams GC. 1957. Pleiotropy, natural selection, and the evolution of senescence. *Evolution* 11:398–411.

Woolfit M. 2009. Effective population size and the rate and pattern of nucleotide substitutions. *Biol Lett.* 5:417–420.

Wright S, Ross H, Jeanette Keeling D, McBride P, Gillman L. 2011. Thermal energy and the rate of genetic evolution in marine fishes. *Evol Ecol*. 25:525–530.

Yang J-N, Seluanov A, Gorbunova V. 2013. Mitochondrial inverted repeats strongly correlate with lifespan: mtDNA inversions and ageing. *PloS ONE.* 8:e73318.

Yang Z. 2007. PAML: a program package for phylogenetic analysis by maximum likelihood. *Mol Biol Evol.* 24:1586–1591.

Table 1. Likelihood ratio test, effect size, mean and standard error of the association between species traits and substitution rates in rockfish. Species traits include body length, maximum lifespan, clutch size, and maximum depth. Since maximum lifespan, clutch size, and maximum depth are all significantly correlated with body length, we also analyse 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 non-protein coding sequences, and the total substitutions across these protein-coding genes and non-protein 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 R^2 for Poisson regression. Likelihood ratio (D) value and the corresponding *P* value in bold indicates the species trait is significantly correlated with the substitution rate, so that their regression coefficient (β) is significantly different from zero. Significance cutoff is 0.05 with Bonferroni correction.

		Mitochondrial					Nuclear				
		dS	dN	dN/dS	Non-protein coding	Total	dS	dN	dN/dS	ITS	Total
Body	D	0.67	0.22	0.12	0.06	1.57	9.15	0.15	1.34	**11.77**	**13.57**
length	*P*	0.41	0.64	0.73	0.81	0.21	2.5×10^{-3}	0.70	0.25	$\mathbf{6.0\times10^{-4}}$	$\mathbf{2.3\times10^{-4}}$
	R^2	0.005	0.008	0.004	0.001	0.009	0.099	0.005	0.035	0.108	0.151
	β	0.1±0.28	0.5±2.01	0.3±1.85	-0.0±0.38	0.1±0.22	-2.0±1.39	-0.4±2.38	1.5±4.03	-1.5±0.94	-1.4±0.82
Maximum	D	**15.07**	2.45	0.94	**14.17**	**32.79**	0.09	4.60	3.18	**19.06**	**19.84**
lifespan	*P*	$\mathbf{1.0\times10^{-4}}$	0.12	0.33	$\mathbf{1.7\times10^{-4}}$	$\mathbf{1.0\times10^{-8}}$	0.76	0.03	0.07	$\mathbf{1.3\times10^{-5}}$	$\mathbf{8.4\times10^{-6}}$
	R^2	0.168	0.097	0.039	0.213	0.313	0.001	0.183	0.111	0.255	0.301
	β	-0.5±0.24	-1.88±1.80	-0.8±1.85	-1.4±0.32	-1.3±0.20	-0.1±0.79	-1.8±2.03	-1.7±2.50	-1.5±0.78	-1.2±0.58
Clutch	D	0.04	0.91	0.86	6.64	0.78	10.00	0.39	2.31	**18.03**	**22.31**

size	*P*	0.84	0.34	0.35	0.01	0.38	1.6×10^{-3}	0.53	0.13	**2.2×10^{-5}**	**2.3×10^{-6}**
	R^2	0.001	0.054	0.050	0.182	0.011	0.219	0.035	0.116	0.354	0.372
	β	0.0±0.11	1.0±1.05	0.4±0.99	-0.5±0.17	-0.1±0.09	-1.4±1.08	-0.3±1.23	1.1±1.66	-0.8±0.42	-0.8±0.39
Maximum	D	1.61	0.42	0.22	2.20	2.63	4.36	1.81	5.18	11.12	4.38
depth	*P*	0.20	0.52	0.65	0.14	0.10	0.04	0.18	0.02	8.5×10^{-4}	0.04
	R^2	0.016	0.016	0.001	0.020	0.018	0.050	0.065	0.120	0.137	0.060
	β	-0.2±0.27	-1.3±2.09	-0.5±1.77	-0.6±0.31	-0.4±0.20	1.0±1.02	-1.0±1.66	-2.0±2.05	-1.6±1.07	-0.7±0.70
Maximum	D	**22.06**	2.47	0.41	6.05	**34.60**	2.08	4.47	6.37	4.70	4.31
lifespan	*P*	**2.6×10^{-6}**	0.12	0.52	0.01	**4.0×10^{-9}**	0.15	0.03	0.01	0.03	0.04
residual	R^2	0.246	0.097	0.017	0.091	0.330	0.024	0.178	0.202	0.063	0.065
	β	-0.5±0.23	-2.1±1.27	-0.4±1.29	-0.8±0.28	-1.2±0.18	0.6±0.81	-1.9±2.14	-2.4±2.57	-0.7±0.67	-0.5±0.54
Clutch	D	10.10	0.01	0.27	6.71	**18.42**	2.13	1.92	3.89	**25.38**	**20.02**
Size	*P*	1.4×10^{-3}	0.92	0.60	0.01	**1.8×10^{-5}**	0.14	0.17	0.05	**4.7×10^{-7}**	**7.7×10^{-6}**
residual	R^2	0.154	0.001	0.015	0.183	0.250	0.047	0.173	0.221	0.499	0.333
	β	-0.3±0.18	0.2±1.44	0.3±1.42	-0.7±0.25	-0.7±0.14	0.8±1.23	-1.1±1.97	-1.9±2.71	-1.5±0.72	-1.1±0.58
Maximum	D	3.88	1.11	0.60	1.02	4.70	10.09	1.36	7.42	1.81	0.03
depth	*P*	0.05	0.29	0.44	0.31	0.03	1.4×10^{-3}	0.24	6.5×10^{-3}	0.18	0.86
residual	R^2	0.038	0.043	0.023	0.009	0.032	0.116	0.049	0.161	0.022	0.000
	β	-0.2±0.26	-2.4±1.96	-0.7±1.84	-0.4±0.29	-0.5±0.19	1.5±1.05	-0.8±1.54	-2.3±1.98	-0.6±0.93	-0.1±0.65

Figure 1. Illustration of the hypothesised 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 the present study. The two observations may connect to each other via 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 because it is positively scaled with body size. 2) If larger species have fewer offspring, they may invest more in DNA repair to avoid costly mutations in the germline; 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 grey 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.

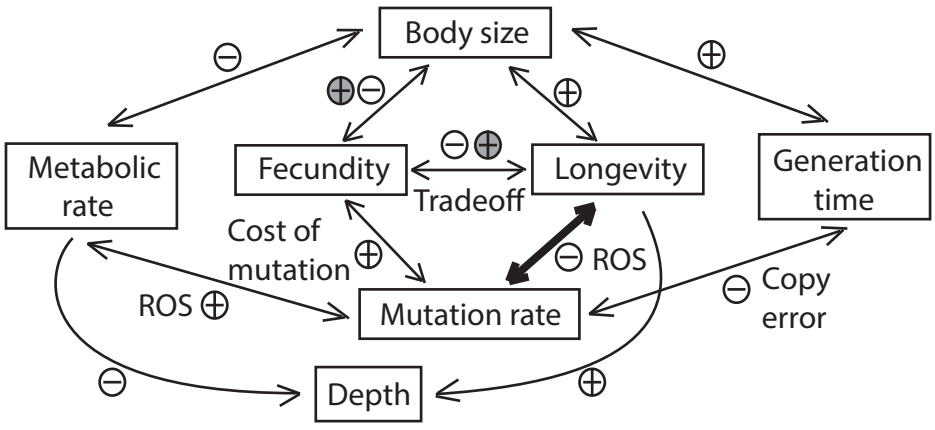

Figure 2. Plots of Welch and Waxman (2008) test for the rockfish dataset and the simulated datasets. A random sample of the simulated datasets with high substituion rates and 30 species pairs are plotted to illustrate that the simulation can reproduce the heteroscedastic patterns in the rockfish mitochondrial synonymous substitution rates, as well as mitochondrial non-protein coding substitution rates and the rate of total substitutions across all mitochondrial sequences. A random sample of the simulated datasets with low substituion rates and 30 species pairs are 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 datasets with 500 species pairs under either high or low substitution rates are plotted to show the heteroscedastic patterns that may not be detected with 30 species pairs.

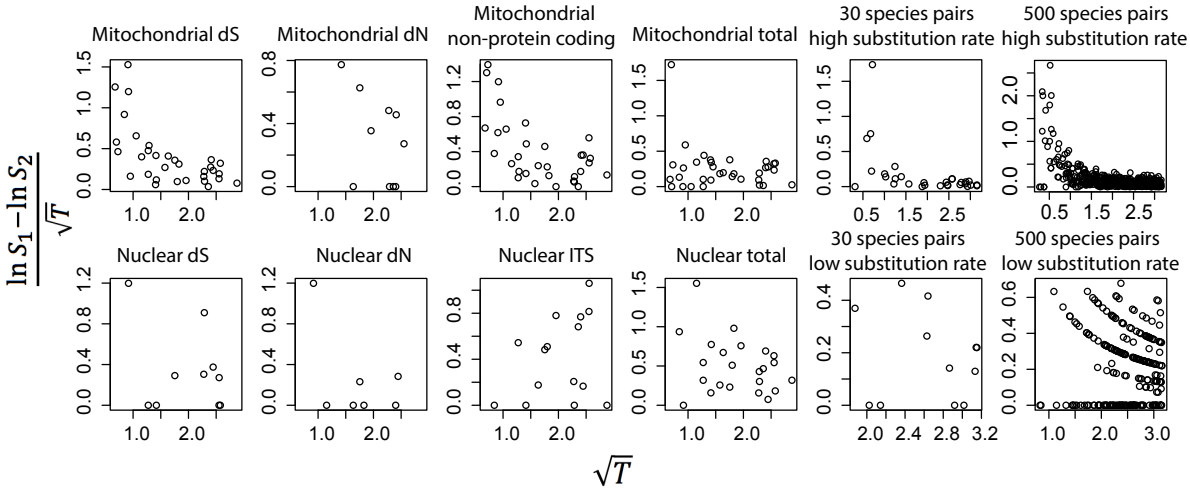

Figure 3. The statistical power of our Poisson regression method and least-squares regression after Welch and Waxman (2008) test. Power is estimated by simulations under each of the combinations of four conditions: 1) number of species pairs, 2) substitution rate: high versus low, 3) rate of evolution of the species trait: fast versus slow, 4) correlation coefficient (r) between substitution rate and species trait. Poisson regression (P) and least-squares regression after the Welch and Waxman test (W) are performed on each of the dataset. For both methods, significance level is set to 0.05. The null hypothesis is no correlation between species trait and substitution rate. Thus the proportion of datasets simulated under no correlation ($r = 0$) that show a significant result (marked as $*$) indicates the type I error rate of the method, which should be around 0.05 (the horizontal line) if the method is well calibrated. The proportion of datasets simulated under correlation that show a significant result (mark as circles for strong correlation and triangles for weak correlation) indicates the power of the method. A method is more powerful if the proportion value is closer to one.

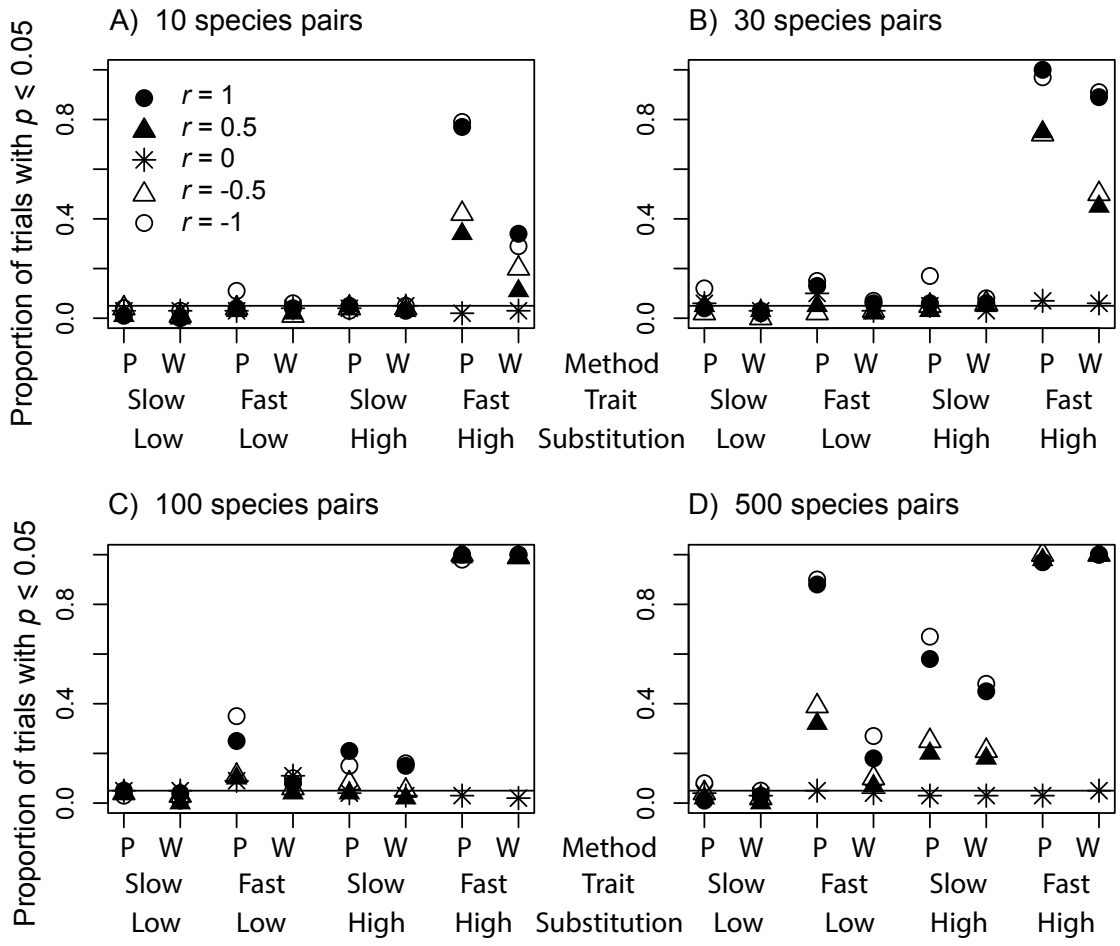

Figure 4. Plots of pairwise correlations among species traits. Each circle represents a rockfish species. All trait values were log-transformed to approach normality.