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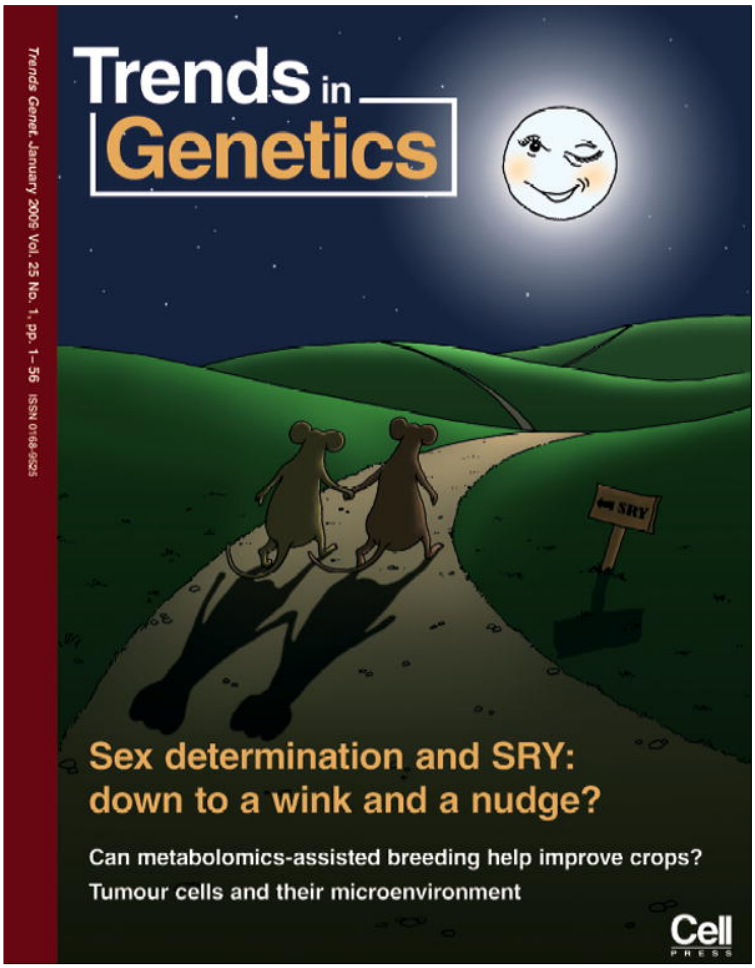

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The need for replication of results

Hunt *et al.* [5] previously reported that dosing mice with 20 μg BPA kg^{-1} for seven days causes chromosome congression failure during oocyte meiosis [5], but – as with any science – it is essential that the results can be independently repeated. Subsequent to Hunt and Hassold's [1] review, three linked papers on BPA were published and in an independent repeat of Hunt *et al.* [5], no effect was seen on congression failure (hyperploidy) [10]. Paccheriotti *et al.* [11] found that acute or chronic dosing of BPA to mice did not induce hyperploidy or polyploidy in oocytes or zygotes, but did see a significant increase of metaphase II oocytes with prematurely separated chromatids after chronic (but not acute) exposure to BPA. There were detrimental effects when oocytes were incubated with doses >7 mg BPA L^{-1} *in vitro* but there were also 'non-linear dose-dependent effects' on meiosis II at lower doses [12]. A failure in replicability has blighted much of the low-dose BPA literature and it is essential to determine which, if any, of these findings are capable of independent replication.

Investigating environmental aneugens is important and worthwhile science; however, the development and deployment of robust, validated and relevant methods is a prerequisite for determining the influence of environmental chemicals on human oogenesis.

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Letters

The evolutionary rate of tuatara revisited

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Often dubbed 'living fossils' (because they seem not to have changed morphologically in >200 million years), tuatara (*Sphenodon punctatus* and *Sphenodon guntheri*) are endemic New Zealand reptiles that are the only extant members of an archaic order of reptiles, which diverged from squamates ~250 million years ago. In a recent paper published in *Trends in Genetics*, Hay and colleagues used ancient DNA to examine the rate of molecular evolution in tuatara and suggested that tuatara have the highest rate of molecular evolution of any vertebrate studied to date [1]. We argue that the serially sampled dataset used by Hay *et al.* [1] is not large enough to estimate an accurate rate of evolution and that their rate is likely to be an overestimate because population structuring was not accounted for in their analysis.

Hay *et al.* [1] concluded that the fast rate of evolution of tuatara is contrary to many predictions about how substitution rates vary with life history traits (e.g. that substitution rates should be lower for ectothermic animals, and those with low metabolic rates [2] or long generation times [3]). The hypervariable region 1 (HVR1) substitution rate for tuatara reported by Hay *et al.* [1] was 1.56 substitutions per site per million years ($ss^{-1}my^{-1}$). However, the 95% highest posterior density (HPD) intervals for this estimate were broad (0.83–2.34 $ss^{-1}my^{-1}$) and overlapped considerably with those of Adelie penguins, aurochs and Mappin's

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moa [1]. Thus, the conclusion of Hay *et al.* [1] that tuatara have the highest rate of molecular evolution in vertebrates is unjustified, because this conclusion is solely based on the point estimate and ignores the associated 95% HPD. If the evolutionary rate presented by Hay *et al.* [1] was converted to a mutation rate by correcting for generation time (mean generation time of tuatara is 40–50 years [4], or 20 000 generations my^{-1}), the estimated mutation rate would be at least an order of magnitude greater than all other published estimates based on heterochronous sequences [5] (e.g. 7.65×10^{-5} ss^{-1}generation^{-1} for tuatara, compared with 3.84×10^{-6} ss^{-1}generation^{-1} for Adelie penguins). This estimate of mutation rate for tuatara is also orders of magnitude higher than mutation rates estimated from pedigrees and mutation accumulation lines [6] and is, thus, implausibly high.

We suggest that the low variability of the dataset combined with the population history of tuatara have biased the HVR1 substitution rate estimate of Hay *et al.* [1] For heterochronous sequences to be useful in estimating the rate of evolution, the population must be 'measurably evolving' [7], that is, characterized by either a high mutation rate or a wide range of sequence sampling times. The tuatara dataset of Hay *et al.* [1] shows very little sequence divergence (mean nucleotide diversity across all samples = 1.9% ± 0.3) and contains only 33 ancient samples. Although these ancient samples span dates 649–8748 years before present (BP), only three were >4000 years old and the majority (22 out of 33) were 1000–3000 years old. When the analysis is re-run with the dates on the ancient samples randomized (Figure 1), the 95% HPD values for the randomized data overlap considerably with those obtained using the original dates and the point estimates for the randomized samples are within the original 95% HPD interval. This indicates that this dataset does not provide sufficient information about the underlying rate of evolution.

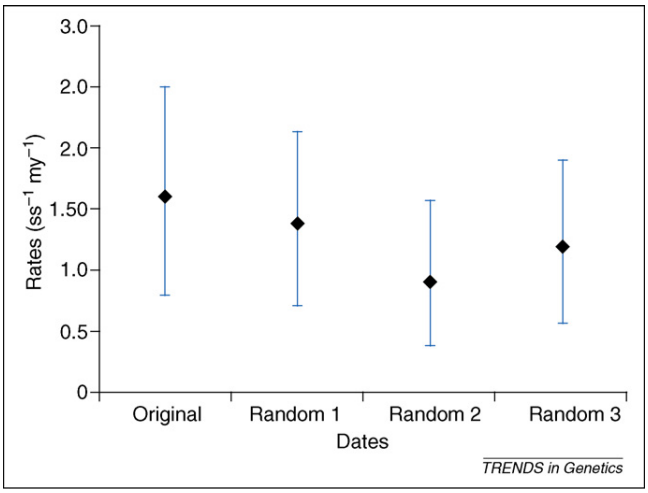

Figure 1. Evolutionary rate estimates obtained when the dates on ancient samples are randomized to destroy any signal obtained from nucleotide changes over time. The dates on the ancient samples were randomized using an Excel macro and the results of three randomizations are shown, along with the original rate. Bayesian Markov-Chain Monte Carlo (MCMC) analyses implemented in the software program Bayesian evolutionary analysis by sampling trees (BEAST; http://beast.bio.ed.ac.uk/) [13], using the model and parameters reported by Hay *et al.* [1] (general time reversible (GTR) + gamma (G) uncorrelated log-normal relaxed clock) were used. Posterior distributions of parameters were sampled every 1000 cycles from the total MCMC chain length of 10 000 000, after discarding the initial burn-in of 1 000 000 steps. This resulted in effective sample sizes >200 for all sampled quantities. Analyses of the dataset using the original dates produced a similar rate estimate in our hands to that reported by Hay *et al.* [1] (1.603 $ss^{-1}my^{-1}$; 95% HPD = 0.782–2.504).

A second confounding influence on the rate estimated by Hay *et al.* [1] relates to the population history of tuatara. The Bayesian methods used here assume no population subdivision [7] and Hay *et al.* [1] used demographic models of either constant population size or exponential growth. None of these assumptions are valid for tuatara over the timescale used by Hay *et al.* [1]. Tuatara were once found throughout the main islands of New Zealand and on many outlying offshore islands. Since the arrival of humans and mammalian predators (~730 years BP [8]), tuatara have experienced a severe population decline and range contraction. Modern natural populations are now restricted to 32 small offshore islands, which are highly genetically structured both between and within islands [9]. We have found that even small breaks in habitat can result in considerable genetic structuring [10], so it is highly unlikely that tuatara have ever existed as a single panmictic population. This structuring is also reflected in mitochondrial DNA (mtDNA) sequences, because virtually every group of islands has a unique set of mtDNA haplotypes and there is a well-supported split between the northern and Cook Strait islands ([11,12] and data from [1]). The ancient and modern samples used by Hay *et al.* [1] were entirely geographically distinct. Modern samples were taken from offshore island populations, whereas ancient samples were taken from mainland New Zealand. Given the level of structuring between the offshore island populations, it is reasonable to expect that a similar level of structuring would be present between mainland and offshore island sites, particularly given the historically small effective population size of most island populations. To account for the possible effect of geographic structuring between ancient and modern samples, we reanalyzed the tuatara dataset using only the ancient samples. The rate we obtained was much lower than the rate of Hay *et al.* [1], being 0.076 $ss^{-1}my^{-1}$ (95% HPD = 0.0016–0.32). We suggest that ancestral population subdivision, unaccounted for by the model, has biased the rate upwards and, thus, a portion of the 'evolutionary rate' estimated for tuatara actually represents geographic differences between ancient and modern samples, rather than temporal differences.

Although Bayesian analyses of ancient DNA datasets provide an exciting new method for estimating rates of evolution, our analysis shows that the population history of the species and the power of the dataset need to be considered before substitution rate estimates should be attempted. We suggest that Hay *et al.*'s [1] claim that tuatara have the highest rate of molecular evolution in vertebrates is open to debate and that accurate estimation of a rate of evolution for tuatara will require a larger dataset from ancient samples and the ability to incorporate population subdivision into the Bayesian model.

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Letters Response

BPA: traditional toxicology testing is inadequate and concerns extend beyond aneuploidy

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In his letter 'Environmental aneugens – the need for replication', Bell takes issue with one aspect of our recent *Trends in Genetics* review [1] on windows of vulnerability during female meiosis. Specifically, his concerns relate to our earlier work linking bisphenol A (BPA) exposure to aneuploidy in mice [2] and to our use of BPA to illustrate how environmental exposures might affect female reproduction. We welcome the opportunity to respond to his criticisms.

Low levels of BPA are detectable in most humans

Bell argues that BPA cannot induce biological effects because rapid metabolism to a non-reactive conjugate results in low circulating levels of biologically active BPA that cannot be measured reliably. However, sensitive and reliable detection methods exist; levels of unconjugated BPA in the several parts per billion (PPB) range have been detected in blood of the majority of individuals studied, with the most sensitive method (isotope dilution gas chromatographic-mass spectrometric measurement; detection limit of 0.01 PPB) reporting mean levels of 4.4 PPB [3]. Thus, the conclusion that biologically active BPA is virtually non-existent is refuted by measurements of unconjugated BPA in humans in numerous studies. Bell further suggests that we know how our bodies metabolize BPA, but the Volkel *et al.* [4] study he cites is the only one that has attempted to directly assess metabolism of known BPA doses in humans. For the most vulnerable humans – fetuses and newborns – there is no information on BPA metabolism, but rapid first pass metabolism of the chemical is likely to be limited or nonexistent at this stage of development. Thus, to dismiss concerns about BPA by arguing that we quickly metabolize it belies our ignorance of BPA metabolism, ignores the many studies that have detected active BPA in human tissues and assumes that babies and infants are the same as adults.

Can we afford to dismiss data from hundreds of studies?

Bell criticizes our statement that low BPA doses elicit adverse effects in mice, citing two studies that show no effects until doses reach 50 mg kg^{-1} day^{-1}. These, and several other studies funded by the American Chemistry Council using traditional toxicology testing paradigms and Good Laboratory Practice (GLP) protocols, have guided the thinking of European and US regulatory agencies, leading to the conclusion that current BPA exposure levels are safe. However, the applicability of this testing paradigm to endocrine disrupting chemicals (EDCs) is disputed and, although the Food Quality Protection Act of 1996 mandated new testing guidelines for EDCs, none currently exist. Importantly, the failure of GLP studies to detect adverse effects of BPA until levels reach the 50 mg kg^{-1} day^{-1} range is at odds with hundreds of low dose studies from academic and government-supported laboratories. [5] Although the use of GLP guidelines implies higher research standards, it is no guarantee of research quality. The Tyl *et al.* [6] study cited by Bell illustrates this point; previous GLP studies were criticized for failing to include positive controls and Tyl and colleagues rectified this by including an estradiol treatment [6]. However, their inability to detect a response to

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