



Molecular evolution of growth hormone and insulin-like growth factor 1 receptors in long-lived, small-bodied mammals



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ABSTRACT

Mammals typically display a robust positive relationship between lifespan and body size. Two groups that deviate markedly from this pattern are bats and African mole-rats, with members of both groups being extremely long-lived given their body size, with the maximum documented lifespan for many species exceeding 20 years. A recent genomics study of the exceptionally long-lived Brandt's bat, *Myotis brandtii* (41 years), suggested that its longevity and small body size may be at least partly attributed to key amino acid substitutions in the transmembrane domains of the receptors of growth hormone (GH) and insulin-like growth factor 1 (IGF1). However, whereas elevated longevity is likely to be common across all 19 bat families, the reported amino acid substitutions were only observed in two closely related bat families. To test the hypothesis that an altered GH/IGF1 axis relates to the longevity of African mole-rats and bats, we compared and analysed the homologous coding gene sequences in genomic and transcriptomic data from 26 bat species, five mole-rats and 38 outgroup species. Phylogenetic analyses of both genes recovered the majority of nodes in the currently accepted species tree with high support. Compared to other clades, such as primates and carnivores, the bats and rodents had longer branch lengths. The single 24 amino acid transmembrane domain of IGF1R was found to be more conserved across mammals compared to that of GHR. Within bats, considerable variation in the transmembrane domain of GHR was found, including a previously unreported deletion in Emballonuridae. The transmembrane domains of rodents were found to be more conserved, with mole-rats lacking uniquely conserved amino acid substitutions. Molecular evolutionary analyses showed that both genes were under purifying selection in bats and mole-rats. Our findings suggest that while the previously documented mutations may confer some additional lifespan to *Myotis* bats, other, as yet unknown, genetic differences are likely to account for the long lifespans observed in many bat and mole-rat species.

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1. Introduction

Understanding the genetic basis of ageing and longevity is of exceptional interest. Typically two main sources of information have shed light on this field; first, the manipulation of specific genes in model organisms that can lead to increased lifespan [e.g. as reviewed in (Kenyon, 2010)], and second, attempts to identify the genetic mutations that have led to increased longevity in natural populations [e.g. (Kim et al., 2011)]. Due to their exceptionally long lifespans, high metabolic

rates and small body sizes, bats have been proposed as potentially underexploited models for ageing studies [e.g. as reviewed in (Brunet-Rossini and Austad, 2004; Wilkinson and South, 2002)]. Although little is known about senescence in bats, it appears that they do not undergo the same typical ageing processes as humans (Brunet-Rossini and Wilkinson, 2009). For example, studies suggest that bats may be able to generate new hair cells within certain regions of the inner ear after birth (Kirkegaard and Jørgensen, 2000), although the functional impact of this on their sensory perception remains unclear. The particular diets of bat species may be either high in fats or sugars, yet bats appear to avoid the associated health implications such as atherosclerosis or hyperglycaemia (Brunet-Rossini and Austad, 2004; Mqokeli and Downs, 2012; Widmaier et al., 1996) which are frequently seen in ageing human populations.

Hypotheses previously put forward to explain bats' long lives include several relating to hibernation, such as altered metabolism and increased predator avoidance [for review see (Brunet-Rossini and

Abbreviations: GH, growth hormone; GHR, growth hormone receptor; IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; SNP, single nucleotide polymorphism.

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Austad, 2004; Wilkinson and South, 2002)]. However, hibernation alone is unlikely to account for the increased lifespan of bats, since not all bats hibernate and hibernation is associated with increased survival across mammals generally (Turbill et al., 2011). The ability to fly has been proposed as leading to greater predator avoidance in bats and volant birds, and thus may at least partly explain their longevity compared to similarly sized non-volant species; in this case extrinsic mortality is reduced which simultaneously drives an increase in lifespan (Healy et al., 2014). Reductions in extrinsic mortality are expected to result in evolutionary adaptation to enhance survival at later life stages (Williams, 1957).

Currently, little is known regarding the genetic basis behind the increased longevity displayed across the ~1300 currently known species of bat. A recent study by Seim et al. (2013) carried out a genomic analysis of Brandt's bat, *Myotis brandtii*, which holds the longevity record for bats, with one male documented to live for 41 years (Podlutzky et al., 2005). This exceptional longevity, coupled with a small body mass (7 g) implies that this species represents the most extreme mammal species outlier in the proposed lifespan body mass relationship [see Fig. 2 from (Podlutzky et al., 2005)]. Seim et al. (2013) found that members of two bat families (Vespertilionidae and Molossidae) shared unique mutations in the transmembrane domains of two genes thought to play crucial roles in growth and ageing: the growth hormone receptor gene (*GHR*) and insulin-like growth factor 1 receptor gene (*IGF1R*). The protein products of these genes are transmembrane receptors found on the surface of mammalian cells, with a single transmembrane region each. *GHR* regulates the cellular effects of growth hormone and *IGF1R* the effects of insulin-like growth factor 1.

Mutations in the genes related to these two hormones and their associated receptors have been linked to several clinical disorders including dwarfism in humans and a long-lived dwarf phenotype in mice (Flurkey et al., 2001; Godowski et al., 1989). Furthermore, genomic evidence from domestic dogs suggests that allelic variation in *IGF1* is responsible for nearly all of the variation in body size found across breeds (Sutter et al., 2007), which in turn is inversely related to breed lifespan (Greer et al., 2007). Additional evidence suggests that a non-synonymous single nucleotide polymorphism (SNP) in *IGF1R* may further contribute to the body size of 'tiny' dog breeds (Hoopes et al., 2012). Single nucleotide polymorphisms in the coding sequence of *IGF1R* in Angus cattle have also been shown to be associated with significant body mass differences in calves (Szewczuk et al., 2013). Such evidence led Seim et al. (2013) to propose that the observed amino acid substitutions in the Vespertilionidae bat transmembrane regions of *GHR* and *IGF1R*, together with traits such as hibernation and low reproductive rate, may contribute to the unusually long lifespan of *Myotis* bat species.

On average bats have a maximum recorded lifespan that is 3.5 times longer than expected given their body size (Wilkinson and South, 2002). Since great longevity is a trait shared by most bats, it is interesting that the documented amino acid changes in bat *GHR* and *IGF1R* were not found in all bat species examined. In particular, the transmembrane domains of larger-bodied fruit-eating bats from the Phyllostomidae and Pteropodidae families were not found to share the same amino acid substitutions seen in *Myotis* bats (Seim et al., 2013). While there is good evidence that the mutations are conserved across *Myotis* bats and closely related species from the same suborder (Yangochiroptera), it is currently unknown whether other long-lived, small-bodied bat species from the other suborder (Yinpterochiroptera), e.g. *Rhinolophus ferrumequinum* – 30.5 years [references within (Wilkinson and South, 2002)], share these mutations.

In addition to bats, African mole-rats (family Bathyergidae) have also been shown to be long-lived for their body size. This is classically illustrated in the naked mole-rat, *Heterocephalus glaber*, which can achieve a maximum lifespan of 31 years with a body mass of 35 g [The AnAge Database: (Tacutu et al., 2013)]. Naked mole-rats have been cited as an example of a mammal that displays negligible senescence [as reviewed in (Buffenstein, 2008)]. They do not undergo

age-related mortality until very late in their lives and breeding females remain fertile and physiologically do not show the typical signs of ageing; for example, decline of vascular system function and increased tumorigenesis (Buffenstein, 2008; Csiszar et al., 2007; Liang et al., 2010). Less is known about the maximum lifespan of many of the other mole-rat species; however, the recorded maximum age of captive *Georychus capensis* is ~5 years (Bennett et al., 2006), while reproductive queens of *Fukomys damarensis* may live >8.5 years (Schmidt et al., 2013). Similarly to bats, reduced extrinsic mortality through predator avoidance has recently been suggested as one possible route to increased longevity in mole-rats, although in this case this is attributed to their fossorial lifestyles (Healy et al., 2014). A high quality genome is available for *H. glaber* (Kim et al., 2011), and through this and related resources, several possible underlying molecular mechanisms underpinning its exceptional lifespan have begun to be documented (Edrey et al., 2012; Kim et al., 2011; Morgan et al., 2013; Yu et al., 2011). However, to date little is known about the molecular evolution of the *GHR* and *IGF1* receptors in the naked mole-rat and closely related rodent species.

To gain further insights into the molecular evolution of these two receptors relating to the genetic control of longevity and body size in bats and mole-rats, we performed phylogenetic analysis of sequence data from each clade, combined with outgroup mammal species. We compared overall substitution rates and selection pressures acting on both of these genes in clades of interest compared to other mammals, and also examined the specific amino acid substitutions that have occurred in the transmembrane region of each gene. Finally, we examined levels of parallel sequence evolution across all pair-wise branch comparisons within the tree (excluding tips) for each gene to test for evidence of molecular convergence between mole-rats and bats.

2. Materials and methods

2.1. Species representation and datasets

We surveyed *GHR* and *IGF1R* nucleotide sequences in 75 mammals, including 26 bats and five mole-rats, generating a total of 64 and 49 sequences for *GHR* and *IGF1R*, respectively. Bat and mole-rat sequences were obtained from a range of sources, including 10 published genomes and five transcriptomes, as well as 17 RNA-seq assemblies of short-read Illumina data assembled with the default parameters of Trinity v. 2013-02-25 (Grabherr et al., 2011). We obtained wide representation of bat species from both the Yinpterochiroptera and Yangochiroptera suborders, including non-echolocating Old World fruit bats and laryngeal echolocating species. From the mole-rats, we obtained sequences from five species which display a range of ecologies and diverse social structures. In addition, to increase the taxonomic sampling we obtained sequences for other divergent subterranean mammals for both genes from RNA-seq assemblies of short-read Illumina data from the East African root rat, *Tachyoryctes splendens*, and *IGF1R* from the golden mole, *Amblysomus hottentotus*.

2.2. Identification of homologous sequences

Complete and partial coding sequences were obtained from genomes and transcriptomes using a BLAST approach (Altschul et al., 1997). For genomic datasets, scaffolds putatively containing genes of interest were initially identified with TBLASTX against the query transcript with an e-value cutoff of $1e^{-6}$ and only keeping hits recovered with >75% identity and with the query sequence in the correct reading frame. Identities were then confirmed by best reciprocal BLAST hits with the same parameters as above. Coding sequences were subsequently extracted from the genomic sequences using BL2SEQ with the query coding sequence. In each case multiple queries were used; human coding sequences (Ensembl IDs: ENSG00000112964 and ENSG00000140443) were initially used in all cases. Additionally, sequences from *Pteropus vampyrus* (Ensembl IDs: ENSPVAG00000005609

and ENSPVAG0000003279), *Myotis lucifugus* (Ensembl ID: ENSMLUG00000017190), and *M. brandtii* (GenBank accession: XM_005875995) were used as subjects for bat searches. For mole-rats, we searched using *H. glaber* coding sequences (GenBank accessions: XM_004848566.1 and XM_004879528). Extracted novel sequences were combined with coding sequences downloaded from Ensembl (Flicek et al., 2013) for all mammalian one-to-one orthologs with the human gene. Ensembl sequences that contained more than 10% missing data were excluded from further analysis. The *M. lucifugus* annotation currently lists *GHR* as a one-to-many ortholog (Ensembl 73); therefore these sequences were excluded from the analysis. Additional mammal sequences were obtained from GenBank, and also the assemblies of short-read Illumina RNA-seq data following the procedure outlined above (see Appendix A: Supplementary Table S1 for species and source information). Novel *GHR* and *IGF1R* sequences have been deposited in GenBank (accession numbers: KM190081–KM190105).

2.3. Alignment and phylogenetic analyses of nucleotide sequences

Nucleotide sequences of each gene were aligned with GUIDANCE (Penn et al., 2010) using the PRANK algorithm (Löytynoja and Goldman, 2005), with codons enforced and 10 bootstraps. Low-quality sequences, those that obtained a quality score of below 0.6, were removed from the multi-fasta file and the alignment recalculated. A Perl script was then used to remove all codon positions from the alignments that contained >50% missing data. This resulted in alignments consisting of 1821 and 3948 base pairs for *GHR* and *IGF1R* respectively. The GTRCAT model was implemented in RAxML v.7.2.8 (Stamatakis, 2006) to produce phylogenies based on each gene alignment, and nodal support for the resultant phylogeny was estimated with 100 bootstraps.

2.4. Examination of amino acid variation in the transmembrane domains

Nucleotide alignments were translated in-frame using the standard genetic code. The 24 amino acids corresponding to the transmembrane domain of each gene (Edens and Talamantes, 1998; Ullrich et al., 1986) were then extracted and sequence variation was examined across the phylogeny. Additionally residue conservation scores were calculated for each column across the entire amino acid alignment in trimAlvs1.4 (Capella-Gutierrez et al., 2009).

2.5. Testing for divergent selection

To test for divergent selection acting on both genes in the two focal clades, bats and mole-rats, the clade model C (Bielawski and Yang, 2004) was run with codeml in PAML v4.4 (Yang, 2007). In this case either the bat or mole-rat clade was set as the foreground clade, and the estimated averaged ω (the number of non-synonymous substitutions per non-synonymous site: the number of synonymous substitutions per synonymous site) of this clade was then compared to that of the averaged ω of the background clade consisting of Laurasiatheria and Euarchontoglires. For each gene, alignments were recalculated, using the above methods, on a pruned taxa set containing only one clade of interest at a time, i.e. when bats were set as the foreground clade of interest, mole-rats were removed from the background dataset and vice versa (see Appendix B: Supplementary data for alignments). The topology of the species tree used was based on recent studies (Agnarsson et al., 2011; Blanga-Kanfi et al., 2009; Faulkes et al., 2004; Meredith et al., 2011; Tsagkogeorga et al., 2013) (Appendix C: Supplementary Fig. S1). Values estimated by each clade model were then compared with model M1a (nearly neutral) via a likelihood ratio test (LRT) with three degrees of freedom (DF), with *P*-values < 0.05 indicating that the alternative model has a significantly better fit compared to the null.

Additionally, we used branch-site models (Zhang et al., 2005) to test for evidence of positive selection acting on three ancestral branches: the common ancestral bat branch, the common ancestral mole-rat branch

and the common ancestral Vespertilionidae + Molossidae branch. In this test, the single branch was set as the foreground and the estimates of site-wise ω values were compared with estimates across the remaining background branches in the phylogeny under model A. This model was compared with the null model A again using a LRT with one DF. The Vespertilionidae + Molossidae branch-site models were carried out on a reduced alignment containing only bat species.

2.6. Quantifying convergent evolution at the amino acid level

For each of the two genes, we characterised the distribution of sequence convergence between pairs of branches in the species phylogeny using the package codeml ancestral (Castoe et al., 2009). We were particularly interested in levels of amino acid convergence between the two focal long-lived clades, bats and mole-rats. The species tree was used to estimate branch lengths and model parameters under the Dayhoff model of amino-acid substitution in codeml in PAML v4.4. These values were then used in codeml ancestral to estimate posterior probabilities of all possible amino-acid substitutions, convergent substitutions (same amino acid) and divergent substitutions, between pair-wise branch comparisons under a Dayhoff model of amino-acid substitution.

3. Results

3.1. Summarising phylogenetic signal in *GHR* and *IGF1R*

Gene sequence alignments of mammalian *GHR* spanned 1821 base pairs (607 amino acids) and that of *IGF1R* consisted of 3948 base pairs (1316 amino acids). Phylogenetic analysis of these nucleotide alignments correctly recovered the majority of the accepted species relationships and major mammalian sub-divisions (Blanga-Kanfi et al., 2009; Meredith et al., 2011; Tsagkogeorga et al., 2013). Trees based on *GHR* and *IGF1R* sequences recovered bats as monophyletic with high (100%) and moderate (79%) bootstrap support, respectively (see Fig. 1). Within bats, the monophyly of the Yangochiroptera sub-division received higher support (100% and 96%) than that of the Yinpterochiroptera (72% and 18%) for *GHR* and *IGF1R*, respectively. Within each suborder the correct familial placements were recovered by *GHR*, but not *IGF1R*, where for example, Phyllostomidae were not recovered as monophyletic. Across taxa, primates typically had the shortest branch-lengths, corresponding to the lowest substitution rates, while rodents and Glires typically had the longest branch-lengths corresponding to a greater number of substitutions. Within bats, Yangochiroptera had the longest branch-lengths; in particular in the *IGF1R* tree, the branch leading to the Vespertilionidae family (*Eptesicus fuscus* + *Myotis* spp.) was the longest branch across the placental mammals surveyed.

3.2. Examination of amino acid variation in the transmembrane domains

Examination of the amino acids corresponding to the transmembrane domains of *GHR* and *IGF1R* indicated that few residues are completely conserved across all mammals (Fig. 1 and Appendix D: Supplementary Fig. S2). Typically, *IGF1R* was seen to be less variable, although fewer sequences were recovered for this gene.

Within bats, the transmembrane region of *GHR* displayed little variation across the six species of Yinpterochiroptera examined (*Eidolon helvum*–*Megaderma lyra* in Fig. 1A), which ranged in adult body mass from 23 to 872 g, and in maximum recorded lifespan from 10 to 30.5 years (see Supplementary Table S1 and Fig. 2). The transmembrane region of *M. lyra* was the most variable, containing two unique amino acid substitutions not seen in any other bat species. Within the suborder Yangochiroptera, considerably more variation was observed with sequence variation supporting three main clades corresponding to Emballonuroidea, Noctilionoidea and Vespertilionoidea (Teeling et al., 2005). We confirmed that the reported deletion at

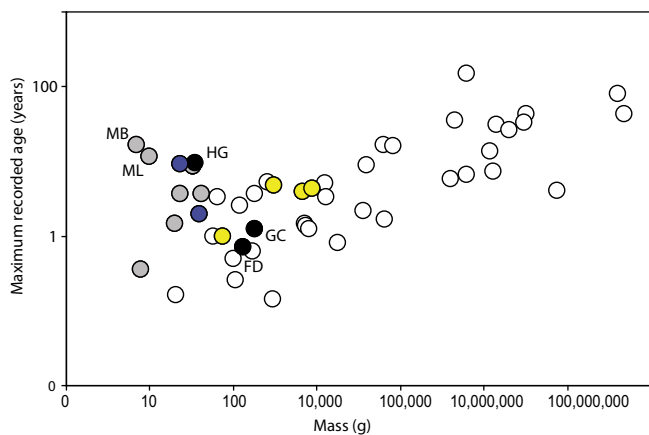


Fig. 2. Scatter plot showing the relationship between maximum recorded lifespan (years) against adult body weight (g) for the species included in this study, data from AnAge (Tacutu et al., 2013); Yanchoiroptera bats (grey points), Old World fruit bats (yellow), echolocating Yinpterochiroptera (blue points), mole-rats (black points) and remaining mammals (white points). GC – *Georchus capensis*, HG – *Heterocephalus glaber*, FD – *Fukomys damarensis*, MB – *Myotis brandtii* and ML – *Myotis lucifugus*.

amino acid deletion (corresponding to codons 522–527 in the human GHR transcript) observed in the guinea pig and mole-rat sequences.

Overall the amino acid sequence of the transmembrane domain of IGF1R was found to be more conserved across all mammals, including most bats, than that of GHR. For example, across Yinpterochiroptera, Mormoopidae and Phyllostomidae, there is little amino acid variation observed (see Fig. 1B). The exception to this pattern is found within Vespertilionidae (*Myotis* spp. and *E. fuscus*), which have a total of eight variable amino acid sites not seen in the other bat species examined. In contrast to bats, the transmembrane region of IGF1R in rodents was found to be highly conserved, with identical amino acids shared between mole-rats and the muroid species (Fig. 1B). Some IGF1R sequence variation was seen in members of Afrotheria, which also display a great range in both lifespan and body mass with the golden mole transmembrane domain displaying considerable sequence variation compared to the remaining species (Fig. 1B).

Amino acid conservation scores for GHR are highly variable across all three (extracellular, transmembrane and cytoplasmic) domains (see Appendix E: Supplementary Fig. S3A). The GHR transmembrane domain does not appear to be remarkable in terms of conservation scores compared to the surrounding non-transmembrane regions. The residues of the extracellular and cytoplasmic domains of IGF1R typically are much higher compared to those of GHR, indicating greater levels of conservation (Supplementary Fig. S3B). However, the IGF1R transmembrane region, together with the ~20 flanking residues, displays much lower conservation levels indicating that this region contains considerable variation across the species included in this study.

3.3. Selection pressures acting on GHR and IGF1R

Clade models of molecular evolution constructed for GHR revealed evidence of significant divergent selection in bats and mole-rats compared to the background clade of carnivores, ungulates, other rodents, Glires and primates (LRT: bats: $P < 0.0001$ and mole-rats: $P < 0.0001$; see Appendix A: Supplementary Table S2A for full results). However, in both model comparisons the estimated ω (dN/dS) value on the foreground clade (FG) and background clade (BG) fell within the range of purifying selection (bats: FG ω = 0.289; BG ω = 0.403; mole-rats: FG ω = 0.489; BG ω = 0.320). Clade models for IGF1R again indicated that the alternative model of divergent selection fit the data significantly better than the null model of neutral evolution for bats and mole-rats (LRT: bats: $P < 0.0001$; mole-rats: $P < 0.0001$); similarly in both cases

the estimated ω (dN/dS) on the foreground clade (FG) and background clade (BG) fell within the range of purifying selection (bats: FG ω = 0.219; BG ω = 0.123; mole-rats: FG ω = 0.102; BG ω = 0.196).

Branch-site tests for positive selection acting on the ancestral bat or ancestral mole-rat branches did not detect any significant positive selection in either GHR (LRT: ancestral bat: $P = 1.00$; ancestral mole-rat: $P = 0.17$) or IGF1R (LRT: ancestral bat: $P = 0.98$; ancestral mole-rat: $P = 1.00$). The majority of sites along each branch were found to be in site-class 0 and therefore, under purifying selection (GHR: ancestral bat: $\omega_0 = 0.106$ and ancestral mole-rat: $\omega_0 = 0.116$; IGF1R: ancestral bat: $\omega_0 = 0.023$ and ancestral mole-rat: $\omega_0 = 0.019$, see Appendix A: Supplementary Table S2B). Despite the levels of sequence variation shown by Vespertilionidae + Molossidae, branch-site models did not detect any significant positive selection acting on the ancestral branch in either gene (LRT: $P = 1.00$ in both GHR and IGF1R; see Appendix A: Supplementary Table S2B), with the majority of sites along each branch found to be under purifying selection ($\omega_0 = 0.101$ and $\omega_0 = 0.020$, in GHR and IGF1R respectively).

3.4. Levels of convergent sequence evolution

Tests for convergent and parallel amino acid substitutions based on branch-wise comparisons were conducted for both genes using the species tree topology. Plots of the summed posterior probability (PP) of total convergent (i.e. both convergent and parallel) substitutions versus summed posterior probability of divergent substitutions across all placental mammal comparisons, excluding any comparisons with a terminal branch, revealed that the majority of substitutions along each gene were divergent (Fig. 3).

In the case of GHR, the ancestral mole-rat branch versus the ancestral Vespertilionidae branch had the highest summed posterior probability of total convergent substitutions among all combinations of mole-rat versus bat branches compared (see Fig. 3A). In total, three sites with a posterior probability of undergoing convergent substitutions >0.20 were detected (Tyr65: PP = 0.67, Cys223: PP = 0.95 and Val404: PP = 0.23).

The second of these substitutions, Cys223, falls within the Fibronectin type 3 domains. The posterior probabilities suggest that these substitutions are most likely to be parallel amino acid changes, i.e. arisen from the same ancestral state.

In comparison, the same analysis conducted for IGF1R revealed no such evidence of parallel substitutions between mole-rats and Vespertilionidae; summed posterior probability of total convergence = 0.002 (see Fig. 3B). Out of all placental mammals sampled, the branch-pair comparison with the highest summed posterior probability of convergence was that between the ancestral Vespertilionidae and the ancestral Old World fruit bat branch; summed posterior probability of total convergence = 9.15. In total, 9 sites with a posterior probability of undergoing convergent substitutions >0.20 were detected (Ala185: PP = 0.97; Gly188: PP = 0.99; Phe678: PP = 1.00; Ile944: PP = 0.99; Ser963: PP = 0.87; Asp964: PP = 0.87; Asp1289: PP = 0.99; Arg1324: PP = 0.99; and Pro1358: PP = 1.00). Once again these were all parallel substitutions.

4. Discussion

4.1. Molecular evolution of GHR and IGF1R in bats and mole-rats

In this study we identified the coding sequence of two hormone receptor genes, GHR and IGF1R, in a comprehensive sampling of 26 bats and five mole-rats. By using a phylogenetic approach, as well as by focusing on individual species, we aimed to test for signatures of molecular adaptation linked to/associated with the increased longevity of these groups. We identified changes unique to members of both groups (for example, a deletion of Phe270 in the transmembrane domain of GHR in emballonurid bats), however, codon-based selection analyses did not identify evidence for positive selection in these focal groups

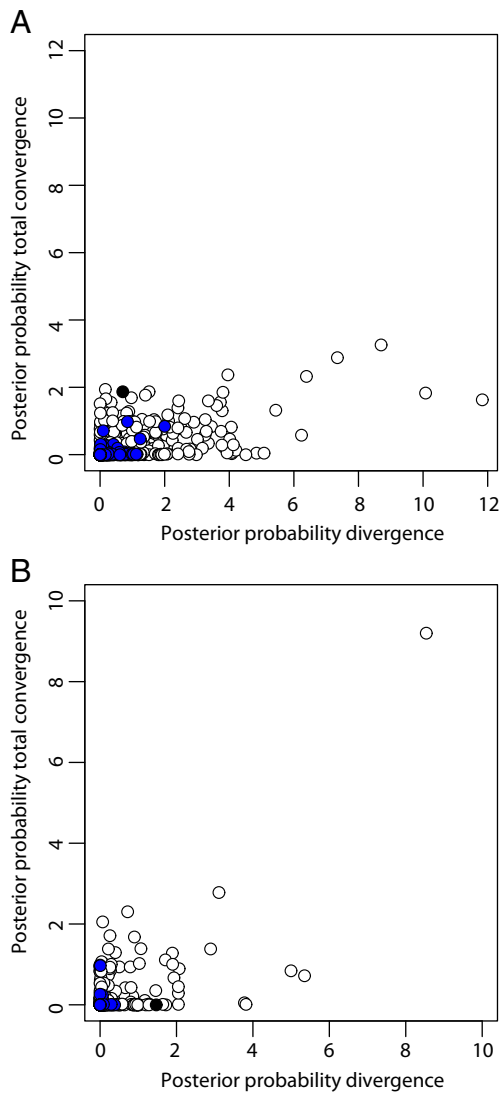


Fig. 3. Plots of the summed posterior probability of total convergent (i.e. both convergent and parallel) substitutions versus summed posterior probability of divergent substitutions across all placental mammal comparisons, excluding any comparisons with a terminal branch for (A) *GHR* and (B) *IGF1R*. Placental mammal pair-wise comparisons (white), mole-rat vs. bat pair-wise comparisons (blue) and ancestral mole-rat vs. ancestral Vespertilionidae (black).

compared to out-group species. Nevertheless, we found statistical support for three amino acids that may have undergone convergent substitutions along the ancestral mole-rat branch and the ancestral Vespertilionidae branch.

4.2. Reconciling *GHR/IGF1R* evolution with life-history traits in bats and mole-rats

Overall we found little evidence to suggest that the transmembrane domains of the *GHR* and *IGF1R* proteins have undergone evolutionary changes across bats that could be linked to their reduced body mass and increased longevity. Despite being the oldest and smallest-bodied Yinpterochiroptera bat examined, with a maximum lifespan of 30.5 years and body mass of 23 g (Wilkinson and South, 2002), the greater horseshoe bat, *R. ferrumequinum* was not seen to possess any unique amino acid substitutions compared to the remaining Yinpterochiroptera in either gene's transmembrane domain. Overall,

the transmembrane domain of the *IGF1R* protein was found to be more highly conserved across all bats and Laurasiatheria, when compared to that of *GHR* – with the exception of the *Myotis* bat branch. Although reliable longevity information was not available for all the *Myotis* bats examined in this study, as a group they are known to be small-bodied and typically long-lived; for example, *M. lucifugus* has a maximum recorded lifespan of 34 years and an adult body mass of around 10 g (Wilkinson and South, 2002). Given the previously documented roles of GH and IGF1 in regulating postnatal growth, it seems unlikely that these two genes alone are responsible for controlling the overall body size of a species. However, it is plausible that the previously documented amino acid substitutions in the transmembrane domains of both *GHR* and *IGF1R* in *Myotis* bats (Seim et al., 2013) may confer a particular functional change in hormonal regulation in these species. In particular, this proposed functional change could relate to the metabolism of these species (especially as they are known to hibernate), although this remains experimentally untested. In general, it seems most likely that increased longevity, coupled with reduced body mass, evolved early in the evolutionary history of bats. Therefore, given that bats show no consistent variation in their *GHR* and *IGF1R* amino acid sequences compared to other mammals, and that most differences appear to have arisen among bat families, it seems doubtful that the molecular evolution of these two hormone receptors has been the principal driving force behind longevity in bats.

Given the wide variation seen in longevity and body mass across rodents, the observed low number of amino acid differences in the two transmembrane domains across the clades is at first somewhat surprising. Nucleotide sequences of *GHR*, however, have been frequently employed as a phylogenetic marker in rodents [for example (Adkins et al., 2001; Galewski et al., 2006)], and this gene is often chosen as it has a relatively low substitution rate thus reducing homoplasy and providing good resolution of taxa (Steppan et al., 2004). This suggests that differences in body mass among rodents are related not to the gene sequence, but to expression or other mechanisms. It has been demonstrated that despite normal expression of *GHR* in guinea pig livers the animals appear to be resistant to the effects of GH, thus suggesting that alternative regulatory pathways may be important (Hull et al., 1996). Whether this is specific to guinea pigs or common to all hystricomorph rodents is currently unknown. In addition to being present as membrane bound dimers, the *GHR* mRNA can undergo proteolytical cleavage in humans and rabbits, or alternative processing in rodents, to generate growth hormone binding protein (GHBP) that circulates in the blood (Edens and Talamantes, 1998; González et al., 2007). In mice and other rodents, GHBP can also be present in a membrane-associated form (González et al., 2007). All three forms bind with GH with a high affinity and so are all likely to play interrelated roles in the regulation of this hormone.

Conflicting evidence exists for the role that *IGF1R* plays in longevity in rodents; for example, female heterozygote knock-out mice live significantly longer than wild-types whereas, male heterozygote knock-out mice do not (Holzenberger et al., 2003). However, no obvious phenotypic traits, such as dwarfism, were observed. A recent study demonstrated a negative correlation between expression levels of *IGF1R* in the brain and longevity across a number of diverse rodent species, including the naked mole-rat (Azpurua et al., 2013). Moreover, because no such relationship was seen in tissue from the heart, lung and kidney, the authors suggested that tissue-specific expression in the nervous tissue may be important to the evolution of longevity in mammals. Despite this, *IGF1R* was not one of the top 20 genes that displayed differential expression in the brain tissue of 2–3 year old naked mole-rats and 6.5 month old mice (Yu et al., 2011). This finding suggests that amino acid changes are not responsible for the increased longevity in African mole-rats, and instead other factors or mechanisms influencing gene regulation may be important, such as differential expression, copy number variation or epigenetic modifications. Alternatively, the increased longevity of mole-rats over muroid rodents may involve any number of alternative genes and/or pathways, potential candidates for which

include those previously identified as displaying differential expression such as *EPCAM*, *SUCLG2* and *EIF4GL* (Yu et al., 2011).

4.3. The wider roles of GHR and IGF1R in mammals

Growth hormone is the major regulator of postnatal growth (Yang et al., 2007), such that sufferers diagnosed with Laron-type dwarfism are typically born with a normal mass and body size (Godowski et al., 1989). This contrasts with insulin-like growth factor 1, which not only is principally involved in the regulation of growth during development, but also affects postnatal growth by interacting with GH. Aberrations in the receptors and associated pathways have been found to be associated with several forms of cancer and growth problems [e.g. (Adams et al., 2000)]. Previous evidence, mainly from mutational studies, suggests that longevity can also be affected – with mutations in the genes relating to these two receptors seen to extend longevity [for example (Junnila et al., 2013; Kaletsky and Murphy, 2010)].

The structure of GHR and IGF1R has been established in several species – both proteins have a single transmembrane domain flanked by extracellular and cytoplasmic domains (Adams et al., 2000; Edens and Talamantes, 1998; Godowski et al., 1989). In order to bind with their respective ligands, the receptors must be present as dimers. Experimental evidence has shown that the IGF1R transmembrane domain is important for activation and function of the receptor (Takahashi et al., 1995). However the GHR transmembrane domain's role in receptor dimerization and activation is debated (Yang et al., 2007). Experimental manipulation of the GHR transmembrane domain amino acid sequence found that its ability to pre-dimerize and therefore, potentially bind with GH, was not significantly affected (Yang et al., 2007). Conversely, numerous mutations in the GHR extracellular domain – which is where the ligand binds – have been shown to result in Laron syndrome [e.g. (Amselem et al., 1993; Pantel et al., 2003)]. Therefore, although previous studies of these receptors in bats (Seim et al., 2013), as well as this current one, have mainly focused on the amino acid variation in the transmembrane domain of GHR the significance of the detected substitutions in this domain remains far from clear. Furthermore we did not detect any sites under positive selection within the extracellular domain of either bats or mole-rats. However, as noted, experimental studies have not been performed on the previously detected bat amino acid substitutions (Seim et al., 2013), so at present any structural and/or functional effects cannot be ruled out.

5. Conclusions

Despite the dramatic variation in lifespan and body size seen across the mammals sampled by this study, both *GHR* and *IGF1R* were found to be under purifying selection in small-bodied, long-lived bats and mole-rats. However, we did detect several examples of family-specific amino acid substitutions in the transmembrane region of bat GHR, which could suggest that this gene may play a role in some aspect of the biology particular to Yangochiroptera. Little amino acid variation was found in the transmembrane domains of either GHR or IGF1R in long-lived mole-rats compared to much shorter-lived rodents. Therefore, evidence suggests that the sequence variation of *GHR* and *IGF1R* does not play a key role in the small body size and longevity seen in bats or mole-rats.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.gene.2014.07.061>.

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