Functional convergence in gastric lysozymes of foregut-fermenting rodents, ruminants, and primates is not attributed to convergent molecular evolution

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Abstract

Convergent evolution is a widespread phenomenon. While there are many examples of convergent evolution at the phenotypic scale, convergence at the molecular level has been more difficult to identify. A classic example of convergent evolution across scales is that of the digestive lysozyme found in ruminants and Colobine monkeys. These herbivorous species rely on foregut fermentation, which has evolved to function more optimally under acidic conditions. Here, we explored if rodents with similar dietary strategies and digestive morphologies have convergently evolved a lysozyme with digestive functions. At the phenotypic level, we find that rodents with bilocular stomach morphologies exhibited a lysozyme that maintained higher relative activities at low pH values, similar to the lysozymes of ruminants and Colobine monkeys. Additionally, the lysozyme of *Peromyscus leucopus* shared a similar predicted protonation state as that observed in previously identified digestive lysozymes. However, we found limited evidence of positive selection acting on the lysozyme gene in foregut-fermenting species and did not identify patterns of convergent molecular evolution in this gene. This study emphasizes that phenotypic convergence need not be the result of convergent genetic modifications, and we encourage further exploration into the mechanisms regulating convergence across biological scales.

Keywords: c-type lysozyme, foregut fermentation, molecular evolution, convergent evolution

Introduction

Charles Darwin famously mused on the "evolution of endless forms most beautiful," suggesting organisms may draw on an unlimited number of evolutionary solutions to overcome environmental obstacles (Darwin, 1859). In contrast, some modern evolutionary biologists suggest that evolution draws on finite options, as evidenced by convergent evolution (Endler, 1986; Schluter, 2000; Vermeij, 2006). Phenotypic convergence in response to similar sources of selection is found across wide environmental and phylogenetic scales (Fleischer et al., 2008; Greenway et al., 2020; Losos, 2009; Parker et al., 2013). However, convergent evolution at one biological scale does not necessitate convergence at others (Dalziel et al., 2017; Diz et al., 2012; Hulsey et al., 2019). In fact, convergence tends to break down at lower levels of biological organization, particularly at the genetic level (Losos, 2011; Manceau et al., 2010), suggesting that organisms may arrive at the same phenotype following unique evolutionary trajectories (Aminetzach et al., 2009; Rosenblum et al., 2010). Given the extensive examples of convergent evolution in nature, identifying the mechanisms that underlie the evolution of similar phenotypes in response to similar selective regimes remains a fundamental goal of evolutionary biology.

Vertebrates' extensive adaptations to specific diet strategies demonstrate the importance of enhancing nutrient acquisition from diverse sources. For example, feeding-structure modifications facilitated dietary specializations in fish (Hulsey et al., 2019; Hulsey and Garcia de Leon, 2005) and myrmecophagous mammals (Reiss, 2000). Additionally, the gastrointestinal structures of vertebrates vary across dietary strategies to maximize nutrient absorption from different sources. For example, the unique structures that facilitate foregut microbial fermentation in herbivorous mammals (Bao et al., 2019; Dehority, 1997; Hume, 1989; Mackie, 2002; Smith et al., 2017) are essential for the digestion of plant materials in these taxa and are considered "key innovations" leading to the success and diversification of these groups (Tran, 2014, 2016). At the molecular level, candidate genes exist

that may make up/be part of/constitute adaptive pathways explaining physiological differences across dietary strategies. For example, the genomes of herbivorous mammals possess more genes associated with detoxification of toxic plant secondary compounds than those of non-herbivorous mammals (Kim et al., 2016), and a recent study investigating dietary adaptations in bats found that frugivory resulted in convergent modification of genes associated with various digestive enzymes (Wang et al., 2020). Despite these examples of convergence in response to diet in animals, few studies have explicitly examined convergent evolution across levels of biological organization in these systems.

The evolution of a digestive lysozyme in foregut-fermenting mammals is a textbook example of convergence that spans levels of organization (Futuyma, 2009). Ruminants and Colobine monkeys feed on similar herbivorous diets, and both taxa exhibit foregut fermentation chambers housing abundant microbial communities that aid in the digestion of plant materials (Dobson et al., 1984; Stewart et al., 1987). Some of these microbes pass into the true stomach along with vegetative material, and these mammals leverage a convergently modified lysozyme to break down the cell walls of bacteria to obtain additional protein (Jollès et al., 1989). Specifically, foregut fermenting mammals have a modified "chicken-type lysozyme" (*Lyz*), an antimicrobial enzyme that usually functions as part of the innate immune system. These lysozymes are abundant in gastric tissues and exhibit unique properties that allow them to function in the acidic compartment of the digestive tract (Dobson et al., 1984; Stewart et al., 1987). In particular, these digestive lysozymes exhibit resistance to pepsin and optimal activity at a narrow and acidic pH range (Dobson et al., 1984; Stewart and Wilson, 1987). They also exhibit amino acid sequence convergence (Jollès et al., 1989; Stewart and Wilson, 1987; Stewart et al., 1987; Swanson et al., 1991), providing evidence that a specific digestive strategy, in this case, foregut-fermentation, may potentiate predictable molecular evolution of a digestive lysozyme.

97 Ruminants and Colobine monkeys are not the only mammals that possess foregut chambers.
98 Rodents with bilocular stomach morphologies— including woodrats (*Neotoma* spp.), deer mice
99 (*Peromyscus* spp.), and voles (*Microtus* spp.) — possess a stomach that is partially separated into
100 proximal and gastric regions, which differ in pH (Carleton, 1973; Kohl et al., 2013). This pH
101 difference may allow for the establishment of symbiotic microbes in the proximal chamber (Toepfer,
102 1891), as observed in woodrats (Kohl et al., 2014; Kohl et al., 2017). The similar gastric morphology
103 and microbial communities of these rodents, ruminants, and Colobine monkeys provide evidence of
104 convergent evolution among these groups. However, no study has investigated whether rodents with
105 bilocular stomach morphology have evolved a digestive lysozyme with similar properties to those
106 expressed in foregut-fermenting mammals.
107 This study aimed to assess functional and molecular convergence of lysozyme across ruminants,
108 colobine monkeys, and rodents. We hypothesized that rodents with bilocular stomachs have
109 convergently evolved digestive lysozymes similar to those seen in foregut fermenters. We predicted
110 that lysozymes extracted from foregut tissues of rodents with bilocular stomachs would possess a
111 narrow range of activity and more acidic pH optima than those of rodents with simpler stomach
112 morphologies. Additionally, we hypothesized that selection for the ability to digest bacterial cells in
113 the gastric stomach has resulted in convergent molecular evolution of lysozyme *Lyz* (chicken-type
114 lysozyme) sequences.
115 Using a comparative phylogenetic approach, we tested for evidence of positive selection in the
116 lysozyme gene of foregut-fermenting ruminants, Colobine monkeys, and rodents. If convergent
117 molecular evolution had occurred, we predicted we would find evidence of positive selection acting
118 on the same codons in rodents with bilocular stomachs as in ruminants and Colobine monkeys. We
119 also/additionally modeled the electrostatic potential of lysozymes to identify differences in surface
120 charges across a range of pH values. Here, we predicted that rodents with bilocular stomachs and

mammals exhibiting foregut fermentation would have a higher ratio of negatively charged to positively charged amino acids (i.e., the (-)/(+) ratio), which a previous study suggested may provide increased stability in digestive lysozymes (Nonaka et al., 2009).

Methods

Lysozyme activity assay

We collected stomach tissue samples from lab-reared Swiss Webster *Mus musculus*, lab-reared *Peromyscus maniculatus* obtained from the Peromyscus Stock Center (Columbia, SC, USA), and wild-caught *Neotoma stephensi* collected from Wupatki, Arizona and housed at the University of Utah (Kohl et al., 2017), with the goal of assessing whether lysozyme differed in pH sensitivity across species with unilocular (*M. musculus*) or bilocular (*P. maniculatus* and *N. stephensi)* stomachs (Kohl et al., 2013). The housing of rodents and collection of tissue was approved by the University of Utah's Animal Care and Use Committee under protocol #12-12,010.

Lysozyme activity was measured following the manufacturer's protocol (LY0100, Sigma-Aldrich, St. Louis, MO, USA). In brief, stomach tissues were homogenized in 2% acetic acid using a handheld homogenizer for 30 seconds (Dobson et al., 1984), centrifuged at 20,000 x g at 4°C for 15 min, and only the supernatant was collected. *Micrococcus lysodeikticus* cells were suspended in reaction buffer to 0.01% (w/v). To assess the effect of pH on lysozyme activity, we created reaction solutions using potassium phosphate buffer (66 mM) and adjusted the pH for each set of reactions. For each reaction, tissue homogenate was added to reaction buffer in 96-well microplates, with three technical replicates per measurement. Immediately following sample addition, the absorbance of these solutions was measured at 450 nm for 10 mins at 37°C (BioTek Instruments Inc., Winooski, VT, USA). The slope of decreasing absorbance over time was used as a measure of lysozyme activity. We calculated relative activity by dividing the activity at each pH by the maximal activity

145 measured for that same individual. Variation in relative lysozyme activity was analyzed using linear
146 mixed-effects models as implemented in the LME4 package (Bates et al., 2015). Species was used as
147 the factor, pH as a covariate, and sample ID was designated as a random effect. Alternative models
148 were assessed using Akaike Information Criteria with finite sample correction (AIC_C) (Johnson and
149 Omland, 2004). The statistical significance of the best-fitting model was determined using a two-way
150 ANOVA, and differences between species across the pH gradient were identified using Tukey's
151 honestly significant difference test.
152
153 *Phylogenetic comparative analysis of lysozyme genes*
154 To gain insights into the molecular evolution of lysozyme in focal rodent species, we collected the
155 lysozyme gene sequences from a diverse set of mammal taxa. We focused on rodents with unilocular
156 (*Mus musculus, M. pahari, M. caroli,* and *Rattus norvegicus*) and bilocular (*Peromyscus maniculatus, P. leucopus,*
157 *Neotoma stephensi, N. lepida,* and *Microtus ochrogaster*) stomachs, primate species that do (*Colobus*
158 *angolensis* and *Piliocobus tephrosceles*) and do not (*Homo sapiens, Nomascus leucogenys, Pan troglodytes, Pan*
159 *paniscus, Pongo abelii,* and *Gorilla gorilla*) exhibit foregut fermentation, Artiodactyla with enhanced (*Bos*
160 *taurus*) and limited (*Camelus dromedarius*) foregut fermentation, as well as a frugivorous bat (*Pteropus*
161 *vampyrus*), omnivorous bear (*Ursus americanus*), a carnivorous felid (*Panthera leo*), and the platypus
162 (*Ornithorhynchus anatinus*) as an outgroup species. The *Neotoma stephensi* lysozyme sequence was
163 generated from a previous study and was identified using a reciprocal BLAST approach (Kohl et al.,
164 2017). The *Neotoma lepida, Peromyscus maniculatus,* and *Peromyscus leucopus* sequences used in this study
165 were retrieved by BLAST using the *Neotoma stephensi* sequence as the query against the available
166 genome data with default parameters (Altschul et al., 1990). All other sequences were acquired from
167 the Ensembl Genome Database (http://www.ensembl.org), and accessions are available in

Supplementary Table 1. Sequence alignments were conducted using MUSCLE (Edgar, 2004) as implemented in MEGAX (Tamura et al., 2011; Supplementary Figure 1).

To establish the phylogenetic framework necessary for conducting these analyses, we used the species tree extracted from the TimeTree database at timetree.org (Hedges et al., 2006). To determine whether the *Lyz* gene was under positive selection in our focal branches (Figure 1), we used branch models implemented in codeml from the PAML package version 4.9a (Yang, 1997). The branch model in codeml estimates variation in the ratio of nonsynonymous-to-synonymous substitutions (ω) for all codons in an alignment across the branches within a phylogeny. We designated each of our focal clades (*Neotoma* woodrats, *Peromyscus* deer mice, *Microtus*, Colobine monkeys, and cow) as foreground clades separately, as well as incorporating a test for all focal species and all cricetid rodents. Using a two-ratio branch model, we calculated a separate ω value for each foreground group (M2; model = 2, NSsites = 0) and compared this to a null model with a single ω estimate for the tree (M0; model = 0, NSsites = 0). A likelihood-ratio test was used to assess the difference between the log-likelihoods of the branch and null models, and statistical significance was determined using a X^2 approximation (Zhang et al., 2005). Similarly, we conducted branch-site tests to evaluate positive selection at specific sites on the branches leading to each lineage (Yang and Nielsen, 2002; Zhang et al., 2005). We estimated false discovery rates for branch and branch-site models using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). In scenarios where sites exhibited evidence of selection, PROVEAN (Protein Variation Effect Analyzer) was used to estimate if amino acid substitutions would result in functional variation of lysozyme.

Protein modeling and protonation

We considered three-dimensional structures of the lysozyme protein from ten species. For three species (*H. sapiens, B. taurus,* and *M. musculus*), solved structures were obtained from the Protein Data Bank: 1C46 (Takano et al., 1999), 1IVM (Obita et al., 2003), and 2Z2F (Akieda, 2011), respectively. The remaining structures were generated via knowledge-based homology modeling using SWISS-Model (Guex et al., 2009; Waterhouse et al., 2018), with the lysozyme structure 133L serving as the template for *Pe. leucopus*, *Pe. maniculatus*, *Ne. lepida*, and *Ne. stephensi*; 1LHL for *R. norvegicus* and *Po. abelii*; and 1GE3 for *C. angolensis* (Biasini et al., 2014). Protonation states were assigned at five different pH levels (2.0, 3.0, 4.0, 5.0, and 7.0) using the PDB2PQR server, which also calculates the approximate partial atomic charge of each protein atom (Dolinsky et al., 2007; Dolinsky et al., 2004; Li et al., 2005). The protonated models were aligned in PyMol, and APBS (Baker et al., 2001) was used to compute electrostatics for each of the structures. Visual Molecular Dynamics (VMD) (Humphrey et al., 1996) was used to model the electrostatics maps and render figures. We then calculated the (-)/(+) ratio of amino acids using the protonation states of lysozyme for each species across pH concentrations.

Results

Lysozyme activity

The relative activity of lysozyme varied by rodent species and pH. The two-way interaction of pH × species best predicted the relative lysozyme activity (Supplementary Table 2), and a two-way ANOVA revealed that this interaction was significant ($p < 0.001$). Observed maximal activity occurred at pH 5.0 and 5.5 in *P. maniculatus* and *Ne. stephensi,* respectively, lower than the observed pH optimum in *M. musculus* (6.0; Figure 2). At the most acidic pH tested, rodents with bilocular stomachs retained higher lysozyme activity when compared to *M. musculus*, but this was only statistically significant in *Pe. maniculatus* (Tukey's honestly significant difference test, $p < 0.01$). These

patterns agree with our prediction that rodents with bilocular stomachs possess lysozymes with greater relative activities at low pH and a more acidic pH optimum. Notably, lysozyme activities in all species exhibited narrow ranges of high activity in our study; mean relative activity exceeded 70% over no more than a 1.0 range of pH values (Figure 2). This finding contradicts our prediction that rodents with unilocular stomachs would have a broader range of moderate to high lysozyme activity across pH concentrations than rodents with bilocular stomachs. Activities in all three species were reduced to similarly low levels at more neutral pH values (Figure 2).

Molecular evolution

Using a molecular-evolution approach, we detected evidence of positive selection on *Lyz* in some lineages. Branch models did not identify differences in nonsynonymous-to-synonymous nucleotide substitution ratios in any lineages (Table 1). This finding deviates from our hypothesis; we predicted branches leading to cricetid rodents, ruminants, and colobine monkeys would exhibit positive selection in the lysozyme gene. However, we found evidence of positive selection at specific codons within the lysozyme gene (Table 2). Cow lysozyme exhibited evidence of positive selection at amino-acid residues 63 and 98 (probability > 0.95 based on Bayes Empirical Bayes analysis; Figure 3). The shift from arginine to histidine at position 98 is predicted to have consequences for lysozyme function based on PROVEAN analyses (score -4.043). Only a single rodent species showed evidence of positive selection at specific sites within the lysozyme gene. Two sites in the *P. leucopus* lysozyme gene also exhibited evidence of positive selection (probability > 0.95; Figure 3). The shifts from arginine to leucine at position 21 and threonine to isoleucine at position 43 both have potential impact on lysozyme activity (PROVEAN scores -4.051 and -3.222, respectively). None of the sites that our methods predicted to be undergoing selection were shared across focal lineages, though a

previous study highlighted site 21 as a convergent shift between ruminants and Colobine monkeys (Stewart and Wilson, 1987).

Electrostatic charge of lysozyme

We identified trends in predicted lysozyme electrostatics across species and pH concentrations. In general, the number of charged residues decreased at lower pH values, as did the (-)/(+) ratio (Supplementary Table 3, Figure 4). At pH 7, *Bos taurus*, *Colobus angolensis*, and *Peromyscus leucopus* lysozymes exhibit high (-)/(+) ratios (Figure 4). In contrast, other species have ratios similar to that of hen egg white lysozyme (Taylor et al., 2019). *P. maniculatus* exhibited a peak in this ratio at pH 4 and was the only species whose ratio was not monotonic with respect to pH (Figure 4).

Discussion

In this study, we investigated whether rodents with bilocular stomachs have convergently evolved a digestive lysozyme with similar properties observed in ruminants and Colobine monkeys. Some species of cricetid rodents have evolved a partially separated stomach morphology in which the proximal stomach houses a microbial community resembling that of other foregut-fermenting mammals (Kohl et al., 2014; Kohl et al., 2017; Kohl et al., 2013). The digestive lysozyme putatively functions to lyse microbial cells passing from the foregut to the gastric chamber, thus liberating microbially produced protein (Stewart and Wilson, 1987; Stewart et al., 1987). We therefore hypothesized that this similar microbial community and gastric morphology might coincide with the evolution of a convergent digestive lysozyme in these rodents.

We found that lysozymes from rodents with bilocular stomach morphologies share some functional characteristics with the lysozymes of other foregut fermenters, but there was no evidence of molecular convergence in the lysozyme gene between other foregut fermenters and these rodents.

These results are similar to those of previous studies that have investigated convergence across levels of biological organization (Fletcher et al., 2001; Natarajan et al., 2016; Natarajan et al., 2015), thus providing further evidence that convergent coding sequence evolution need not underlie convergent phenotypes.

Differences in lysozyme activity

Our study specifically measured the relative enzymatic activity of lysozyme from rodents across a pH gradient to assess if lysozymes of rodents with bilocular stomachs have functional properties similar to those of ruminants and Colobine monkeys. We found that *Neotoma stephensi* and *Peromyscus maniculatus* lysozymes exhibited lower pH optima than the *Mus musculus* lysozyme (Figure 2). Indeed, rodents with bilocular stomach anatomies had higher lysozyme activities at pH 5.0, indicating that their enzymes retain function at lower pH values than those from species with unilocular stomachs. Maximum lysozyme activities for *P. maniculatus* and *N. stephensi* occurred at pH 5.0 and 5.5, respectively, similar to the peak activities observed in studies of other foregut-fermenting mammals (pH ~5.0), including cow, sheep, black-tailed deer, and langur monkeys (Dobson et al., 1984; Stewart and Wilson, 1987). It is possible that the optima of these lysozymes, particularly that of *P. maniculatus,* may occur at even lower pH values, which should be tested in future studies. Altogether, these findings support our prediction that rodents with bilocular stomachs maintain higher lysozyme activities at low pH level.

There was no difference in the range of activity among rodent species. We predicted that rodents with unilocular stomachs would possess lysozymes that function across broad pH ranges, like those of pigs and chickens (Dobson et al., 1984; Stewart and Wilson, 1987); however, we observed that all rodents, including *M. musculus*, exhibited optimal activity in a narrow range. One possible explanation for the narrow range of lysozyme activity observed in *M. musculus* is that this

286 species exhibits multiple c-type lysozyme genes that arose via gene duplication and may differ in
287 activity. The lysozyme sequences analyzed in this study were obtained based on sequence similarity
288 to *Neotoma stephensi*, which was previously identified based on similarity to that of the bovine
289 digestive lysozyme. While we are confident in these comparisons, future investigations should
290 consider the possibility of multiple lysozyme genes possessing unique functions in rodents.
291
292 *Molecular evolution*
293 Although we identified differences in functional aspects of lysozyme activity, these measures were
294 not linked to convergent molecular evolution at the level of individual amino acids. Previous
295 investigations into the convergent molecular evolution of lysozyme in ruminants and Colobine
296 monkeys identified seven amino-acid substitutions shared between these taxa (Jollès et al., 1989;
297 Stewart and Wilson, 1987; Stewart et al., 1987; Swanson et al., 1991). Our study aimed to assess
298 evidence for positive selection on these and other sites across previously studied foregut-fermenting
299 mammals and rodents with bilocular stomachs. While there was no evidence suggesting selection
300 along branches, there were two instances where evidence of selection at branch-sites was observed –
301 the *Bos taurus* and *Peromyscus leucopus LYZ* gene.
302 The lysozyme sequence of only one rodent species exhibited evidence of positive selection – *P.*
303 *leucopus* at sites 21 and 43. At site 21, the *P. leucopus LYZ* sequences encodes a leucine instead of an
304 arginine (Figure 3). Interestingly, this same site is often shifted from an arginine to lysine in
305 ruminants and Colobine monkeys, and this substitution is thought to be partially responsible for the
306 digestive function of lysozyme (Stewart and Wilson, 1987). This change may explain the putatively
307 functional consequence this substitution has on lysozyme activity as suggested by the PROVEAN
308 results. Additionally, two sites with evidence of positive selection in the cow had shifts from arginine
309 to either lysine or histidine. A previous study suggested that substitutions in the digestive lysozyme

310 gene of ruminants and monkeys favored substit of arginine for lysine, which could potentially
311 increase stability and function in the digestive tract (Stewart and Wilson, 1987). At one of these sites,
312 position 14, four species of foregut-fermenting rodents possess a serine instead of an arginine,
313 representing a transition to an uncharged polar amino acid from a positive one along the ancestral
314 branch leading to *Peromyscus* and *Neotoma* species. Although cricetid rodents and other foregut-
315 fermenting mammals did not share the same amino acid substitution, the observation of arginine
316 replacement at this same site across foregut fermenting mammals and rodents suggests that this
317 substitution may have consequences for protein structure and function (Sokalingam et al., 2012).
318 Overall, our results suggest that selection does not produce convergent evolution at the lysozyme
319 sequence level in our study.
320 Interestingly, three of the seven sites with putatively adaptive convergent amino-acid
321 substitutions in Colobine monkeys and ruminants (positions 14, 21, and 50) also have substitutions
322 in at least one rodent taxon with bilocular stomach morphology (Figure 3). Although these sites did
323 not show strong evidence for selection or convergence in our analyses, they may play an important
324 role in mediating lysozyme function in the digestive tract. Future work investigating the mechanisms
325 underlying variation in lysozyme activity could include site-directed mutagenesis akin to previous
326 studies investigating the catalytic site of lysozyme (Malcolm et al., 1989).
327
328 *Lysozyme structure and electrostatics*
329 Our electrostatic models yielded insights into the potential protein chemistry of lysozyme function.
330 By modeling the electrostatic charges across a range of pH values, we determined that the ratio of
331 negatively to positively charged amino acids [i.e., the (-)/(+) ratio] at pH 7 was greater in previously
332 identified foregut fermenters and *Peromyscus leucopus* than in the other species (Figure 4). Additionally,
333 these species and *P. maniculatus* exhibit marginally higher (-)/(+) ratios at a physiologically relevant

pH (4.0). In contrast, other species exhibit a similar ratio to that observed in hen egg white lysozyme (Taylor et al., 2019). While the functional significance of these differences is unknown, it has been previously suggested that the charges of this enzyme are important given that the peptidoglycan substrate of lysozyme is also a charged molecule (Taylor et al., 2019). The increased negative charge may also increase structural stability in more acidic environments (Nonaka et al., 2009).

Convergent evolution across levels of organization

The extent to which convergent modifications at the molecular level regulate phenotypic patterns of convergence remains an open question in evolutionary biology. While there are examples of convergent molecular evolution leading to convergence in phenotypes (Dobson et al., 1984; Feldman et al., 2012; Greenway et al., 2020; Jollès et al., 1989; Stewart and Wilson, 1987; Stewart et al., 1987; Swanson et al., 1991), an equal number of examples suggest phenotypic convergence is not always the result of similar molecular changes (Chen et al., 2020; Corbett-Detig et al., 2020; Steiner et al., 2008). For example, some high-altitude birds have evolved hemoglobin with similar functional properties that do not share the same underlying genetic changes (Natarajan et al., 2015; Natarajan et al., 2016). The lysozymes of foregut-fermenting rodents provide another example; they have enzymatic activity at low pH concentrations, as seen in other foregut-fermenting mammals, despite showing no detectable molecular convergence.

The presence of functional convergence in the absence of convergence at the genetic level begets the question: under what circumstances does convergent evolution occur at both the molecular and phenotypic levels? Previous studies have highlighted that molecular convergence is most likely when evolutionary solutions are constrained by environmental effects on specific biochemical and physiological pathways (Barts et al., 2018; Greenway et al., 2020; Reid et al., 2016). Perhaps convergent evolution of a digestive lysozyme can be mediated by the recruitment of

alternative isoforms of the same gene or by coopting the function of a different lysozyme gene, making molecular evolution more difficult to predict (German et al., 2015). Alternatively, perhaps the presence of amino-acid substitutions at the same sites across rodents, ruminants, and Colobine monkeys indicates that modification of these sites away from the ancestral state alters lysozyme function, similar to what has been observed in the evolution of coloration in White Sands lizards (Rosenblum et al., 2010). Broadly, investigating whether molecular convergence underlies phenotypic convergence sheds light on the mechanisms regulating adaptive evolution under different selective regimes.

Conclusions

Overall, this study highlights how convergent molecular modification need not underlie functional convergence in organisms exposed to similar sources of selection. To our knowledge, this is the first study to recognize functional similarities between the lysozymes of previously studied foregut-fermenting mammals and rodents with bilocular stomach morphologies. Ruminants, Colobine monkeys, *Neotoma stephensi,* and *Peromyscus maniculatus* possess convergent lysozyme functional activity, but we did not detect molecular mechanisms that clearly underlie these functional shifts. It is important to consider that our analyses were conducted on coding sequences, and the variation in function we observed may be the result of selection on the cumulative effects of post-transcriptional, post-translational, or other gene-regulatory processes. Given that molecular convergence does not always underlie phenotypic convergence in nature, the field of evolutionary biology needs to increase comprehensive studies to identify the mechanisms regulating convergence across levels of biological organization.

382

383 **Ethics Approval**

384 The housing of rodents and collection of tissue was approved by the University of Utah's Animal

385 Care and Use Committee under protocol #12-12,010.

386

387 **Data Availability**

388 All data and code associated with physiological and molecular evolution analyses are available on

389 GitHub (http://github.com/nickrbarts/lysozyme_convergence).

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Tables

Table 1: Results of branch tests evaluating differences in nonsynonymous-to-synonymous amino-acid substitution rates between foreground (foregut-fermenting mammals and putative foregut-fermenting rodents) and background lineages.

Clades	$\mathbf{lnL_{null}}$	$\mathbf{lnL_{branch}}$	$\mathbf{2\Delta lnL}$	P	(i/m)Q	$\boldsymbol{\omega}_{\mathbf{null}}$	$\boldsymbol{\omega}_{\mathbf{background}}$	$\boldsymbol{\omega}_{\mathbf{foreground}}$
All Foregut Fermenters	-3252.4	-3249.2	6.384	0.012	0.007	0.30319	0.28865	0.57633
Bos taurus	-3252.4	-3251.6	1.597	0.206	0.029	0.30319	0.31943	0.54835
Colobine monkeys	-3252.4	-3249.4	5.889	0.015	0.014	0.30319	0.29190	1.42036
All Cricetid rodents	-3252.4	-3252.3	0.208	0.647	0.050	0.30319	0.30150	0.46276
Microtus ochrogaster	-3252.4	-3251.9	0.809	0.368	0.035	0.30319	0.33942	0.13840
Neotoma woodrats	-3252.4	-3252.2	0.269	0.604	0.043	0.30319	0.30620	0.14211
Peromyscus mice	-3252.4	-3250.8	3.194	0.074	0.021	0.30319	0.31059	0.09749

Table 2: Results of branch-site tests evaluating positive selection affecting a subset of codons between foreground (foregut-fermenting mammals and cricetid rodents) and background lineages. Each lineage was analyzed separately to account for potential variation, except for the colobine monkeys, who had indistinguishable sequences. Lines in bold indicate taxa with evidence for sites under selection.

Lineage	$\ln L_{null}$	$\ln L_{branch\text{-}site}$	$2\Delta \ln L$	P	(i/m)Q
All foregut fermenters	-3118.518	-3115.555	5.927	0.015	0.015
Bos taurus	**-3120.471**	**-3116.3**	**8.354**	**0.004**	**0.005**
Colobine monkeys	-3124.421	-3124.2	0.517	0.472	0.020
All cricetid rodents	-3125.671	-3125.671	0.000	1.000	0.055
Neotoma sp.	-3125.431	-3125.431	0.000	1.000	0.045
Peromyscus sp.	-3125.671	-3125.671	0.000	1.000	0.050
Microtus ochrogaster	-3125.671	-3125.671	0.000	1.000	0.030
Neotoma lepida	-3125.671	-3125.671	0.000	1.000	0.035
Neotoma stephensi	-3125.671	-3125.671	0.000	1.000	0.040
Peromyscus maniculatus	-3125.671	-3125.671	0.000	1.000	0.025
Peromyscus leucopus	**-3124.602**	**-3120.964**	**7.277**	**0.007**	**0.010**

Figures

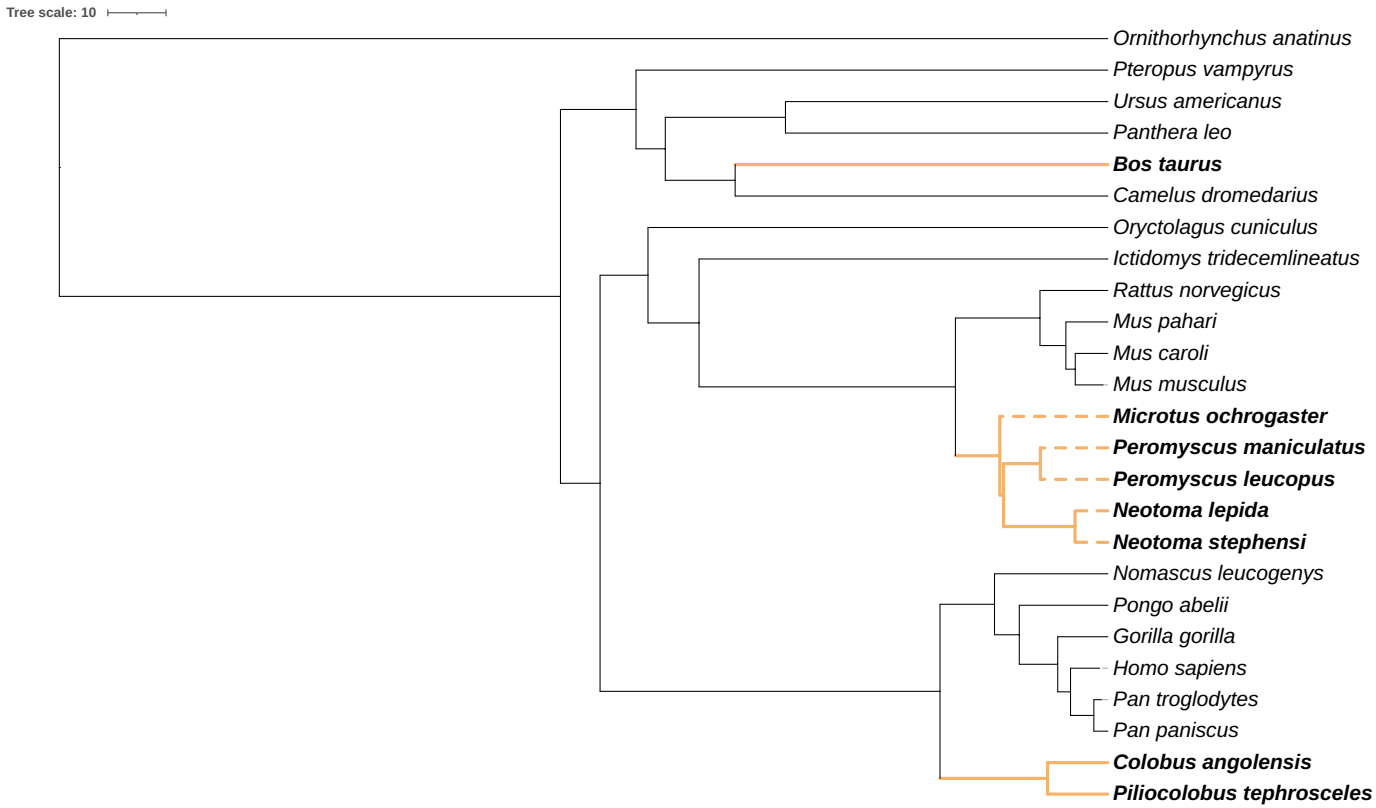

Figure 1: Cladogram extracted for all species from the TimeTree database at timetree.org (Hedges et al., 2006). Previously identified foregut fermenters (*Bos taurus, Colobus angolensis,* and *Piliocobus tephrosceles*) as well as hypothesized foregut-fermenting rodents (*Microtus ochorogaster, Peromyscus leucopus*, *P. maniculatus*, *Neotoma lepida,* and *N. stephensi*) are highlighted in orange. Branches with dotted lines indicate that both individual species as well as clade were included in branch-site models.

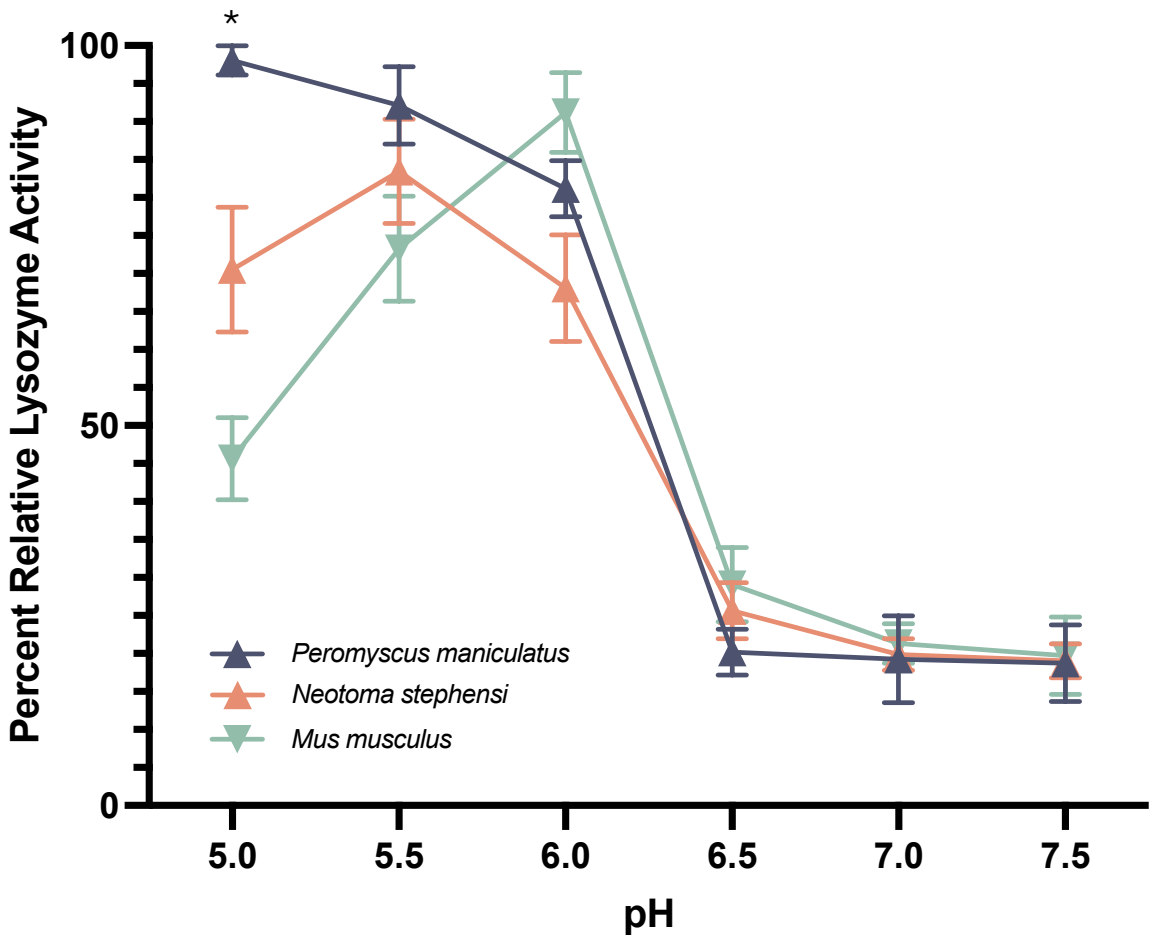

Figure 2: Relative lysozyme activities of from *Mus musculus Peromyscus maniculatus*, and *Neotoma stephensi*. Points represent mean ± standard error of relative activity, calculated by dividing the activity at each pH by the maximal activity measured for that same individual. ▼ indicates a rodent with unilocular stomachs, and ▲ indicates rodents with bilocular stomachs. Asterisk indicates statistically significant difference (*P. maniculatus* lysozyme activity > *M. musculus* ($p < 0.05$).

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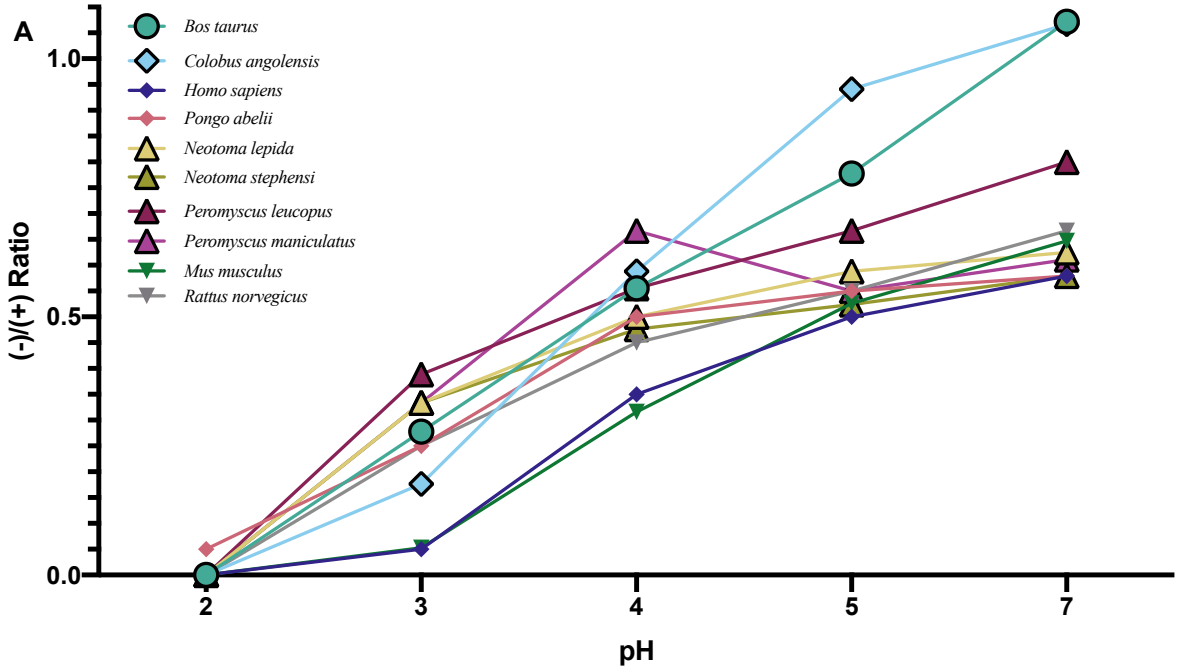
Species	14	17	21	43	50	63	75	87	98	101
Ornithorhyncus anatinus	Q	M	R	T	R	Y	N	D	R	R
Oryctolagus cuniculus	K	L	K	T	Q	Y	N	D	R	R
Ictidomys tridecimlineatus	R	M	H	T	Q	Y	N	D	R	R
Rattus norvegicus	R	M	Y	R	Q	Y	N	D	R	R
Mus pahari	R	M	R	T	R	Y	N	D	R	R
Mus musculus	R	M	Y	T	Q	Y	N	D	R	R
Mus caroli	R	M	Y	T	Q	Y	N	D	R	R
Microtus ochrogaster	R	M	R	T	K	Y	N	D	R	R
Neotoma lepida	S	M	R	T	R	Y	N	D	R	R
Neostoma stephensi	S	M	R	T	R	Y	N	D	R	R
Peromyscus maniculatus	S	M	R	T	Q	Y	N	D	R	R
Peromyscus leucopus	S	M	**L**	**I**	Q	Y	N	D	R	R
Pteropus vampyrus	R	M	K	T	K	Y	N	D	R	R
Ursus americanus	R	L	K	T	R	Y	N	D	R	R
Panthera leo	K	M	K	T	R	Y	N	D	R	R
Camelus dromedarius	K	M	R	T	G	Y	N	D	R	R
Bos taurus	K	L	K	T	E	**W**	D	N	**H**	S
Colobus angolensis	K	L	K	T	E	Y	D	N	R	S
Piliocobus tephrosceles	K	L	K	T	E	Y	D	N	R	S
Nomascus leucogenys	R	M	R	T	R	Y	N	D	R	R
Pongo abelii	R	M	R	T	R	Y	N	D	R	R
Pan paniscus	R	M	R	T	R	Y	N	D	R	R
Pan troglodytes	R	M	R	T	R	Y	N	D	R	R
Homo sapiens	R	M	R	T	R	Y	N	D	R	R
Gorilla gorilla	R	M	R	T	R	Y	N	D	R	R

625 **Figure 3:** Alignment of *LYZ* sequence showing amino-acid positions that display evidence of
626 selection in at least one lineage used in our study (black) or previously determined to contain
627 convergent substitutions in ruminants and Colobine monkeys (red) (Stewart and Wilson, 1987).
628 Focal lineages are in boxes, and the sites predicted to be under selection are in bold.


A
Bos taurus
Colobus angolensis
Homo sapiens
Pongo abelii
Neotoma lepida
Neotoma stephensi
Peromyscus leucopus
Peromyscus maniculatus
Mus musculus
Rattus norvegicus
(-)/(+) Ratio
1.0
0.5
0.0
2
3
4
5
7
pH

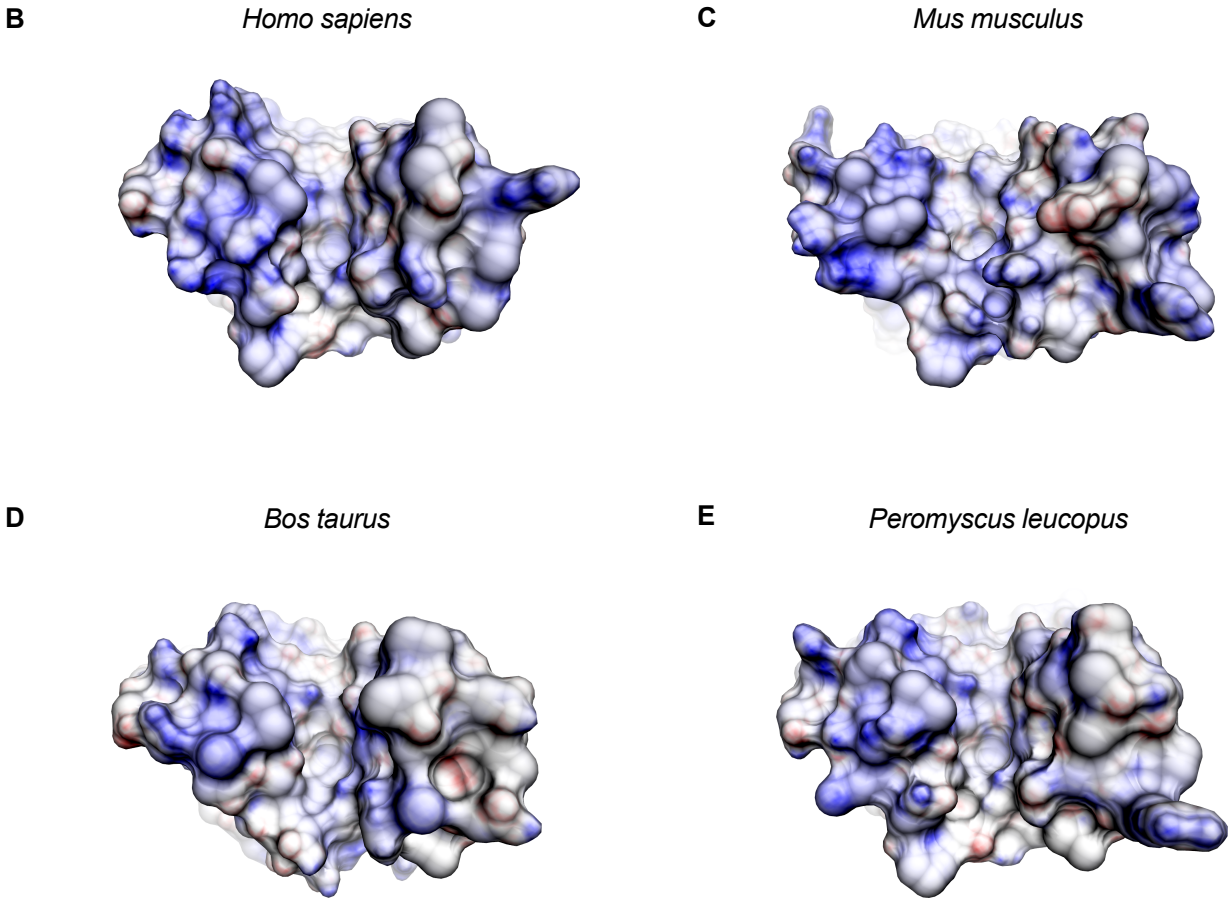

B
Homo sapiens
C
Mus musculus
D
Bos taurus
E
Peromyscus leucopus

Figure 4: A: Predicted changes in the ratio of negatively charged to positively charged residues in lysozymes across a gradient of pH values. ●: Ruminants; ▲: Rodents with bilocular stomach morphology; ▼: Rodents with unilocular stomach morphology; ♦: Primates. Symbols with bolded outlines indicate foregut-fermenting taxa. **B-E:** Lysozyme models colored according to the electrostatic potential at the protein surface (pH 4.0) from *Homo sapiens, Mus musculus, Bos taurus,* and *Peromyscus leucopus*, respectively. Red coloration indicates a negative charge, and blue coloration indicates a positive charge.