Convergent molecular constraint in mammalian carnivores: Selection on liver and kidney functions during the evolution of more carnivorous diets

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ABSTRACT

Background

Mammalia comprises a great diversity of diet types and associated adaptations. An understanding of the genomic mechanisms underlying these adaptations may offer insights for improving human health. Comparative genomic studies of diet that employ taxonomically restricted analyses or simplified diet classifications may suffer reduced power to detect molecular convergence associated with diet evolution. Here, we used a quantitative carnivory score and 80 mammalian species to detect significant correlations between the relative evolutionary rates of genes and changes in diet. Increases in this carnivory score indicated increasing amounts of animal protein in a species' diet.

Results

We identified six genes—*ACADSB*, *CLDN16*, *CPB1*, *PNLIP*, *SLC13A2*, and *SLC14A2*—that experienced significant evolutionary constraint and greater functional importance as increasingly carnivorous diets evolved. We further considered the biological functions associated with diet evolution and observed that pathways related to amino acid and lipid metabolism, biological oxidation, and small molecule transport were evolutionarily constrained in association with increased carnivory. Liver and kidney functions showed a similar pattern of greater constraint as carnivory increases.

Conclusions

Our results indicate that, during adaptation to increasing carnivory, selection acts on the liver and kidneys to maintain sufficient metabolism and excretion of substances found in excess in carnivorous diets.

KEYWORDS

adaptation, carnivory, convergent evolution, diet, mammal, metabolism

BACKGROUND

As a diversity of mammalian diets arose from the ancestral insectivorous strategy (1), numerous physiological, morphological, and behavioral adaptations also evolved. Understanding the mechanisms of these adaptations may lead to improvements in human health. For example, glucose metabolism in healthy carnivores resembles diabetes in humans and other non-carnivores (2). Polar bears are adapted to persistently high blood cholesterol levels (3), which have been implicated in human cardiovascular disease (4), and some networks underlying polar bear adaptation have been implicated in high fat diet adaptation in humans (5). Thus, comparative genomic studies of diet offer novel insights for medical advancement. Large-scale comparative genomic resources, consisting of data from hundreds of non-model organisms, have been developed to support such research (6, 7).

Previous studies identified many genomic changes accompanying adaptations to carnivorous and herbivorous diets. For example, the convergent evolution of herbivory in the giant and red pandas coincided with adaptive molecular evolution of genes associated with the utilization of nutrients that are scarce in bamboo, as well as limb development genes that facilitated growth of the pseudothumb (8). In cetaceans, positive selection for proteinases and lipases and loss of pancreatic *RNASE1* expression have been attributed to the evolution of carnivory from a herbivorous ancestor (9). Loss of the hormone-receptor pair *INSL5-RXFP4*, which regulates appetite and glucose homeostasis, is thought to be an adaptation to irregular feeding patterns in carnivores (10). Positive selection in carnivores acted on genes associated with successful hunting—such as muscle strength and agility—as well as digestion of fat- and protein-rich foods (11).

While previous studies provided valuable insights into lineage-specific dietary adaptations, studies that incorporate data encompassing a larger number of taxa can identify mechanisms shared across convergent changes in diet. To date, comparative genomic studies that sampled broadly within mammals have used coarse dietary classification systems, such as herbivore, carnivore, and omnivore (10-12). This results in a loss of important dietary information, as species grouped together differ in the proportions of plant and animal matter consumed and may show divergent adaptations (13-16). Thus, the power of comparative genomic studies that investigate the molecular mechanisms of dietary adaptation may be reduced.

The objectives of our study are two-fold. First, we identify genes that are important for convergent adaptation to increasingly carnivorous or herbivorous diets in mammals. We accomplish this by detecting significant correlations between gene evolutionary rate shifts and changes in a continuous diet score mapped across a phylogeny of 80 species. Second, we use convergent evolutionary rate shifts to elucidate the biological functions associated with carnivorous and herbivorous adaptation across Mammalia. Our analyses encompass unprecedented taxonomic breadth and dietary nuance compared to previous comparative genomic studies of diet in mammals.

METHODS

Preparation of dietary data

To create a continuous categorization of diet, we obtained data from the EltonTraits mammal dataset (17). Data compiled in EltonTraits came primarily from Walker's Mammals of the World (18), which describes species' diets based on summaries of existing literature. Qualitative descriptions of dietary preferences were translated by the EltonTraits authors into numerical values representing the proportion of diet comprising numerous food types. In this study, we generated a carnivory score for each species by summing the proportions of vertebrates and invertebrates in their diet. Thus, our study treats invertivorous species as carnivorous. A score of 0 represents a completely herbivorous species, and a score of 100 represents a completely carnivorous animal. Due to the structure of EltonTraits, carnivory scores jumped in steps of 10 from 0 to 100 (Fig. 1; Additional File 1: Table S1); thus, we analyzed 11 ordinal bins which we hereafter call degree of carnivory.

Study species and gene alignments

For our analyses of relative evolutionary rates (RERs), we selected 80 species that encompass the taxonomic and dietary breadth of therian mammals (Fig. 1; Additional File 1: Table S1). We pruned multiple codon alignments generated with TOGA by the Zoonomia Consortium (6, 19) so that they included only our chosen species. We then filtered the alignments to remove sequences not covering 50% of the total aligned sequence length. For alignments that included more than one sequence per species, we retained the sequence with the highest percent identity to human. We only analyzed alignments with sequences that survived filtering for at least 60 target species. Our filtering reduced the number of genes analyzed from 17,439 to 15,117. An overrepresentation

analysis of Gene Ontology (GO) terms indicated that the unanalyzed genes were predominantly associated with DNA assembly and organization, perception of chemical stimuli, and gene silencing (Additional File 1: Table S2).

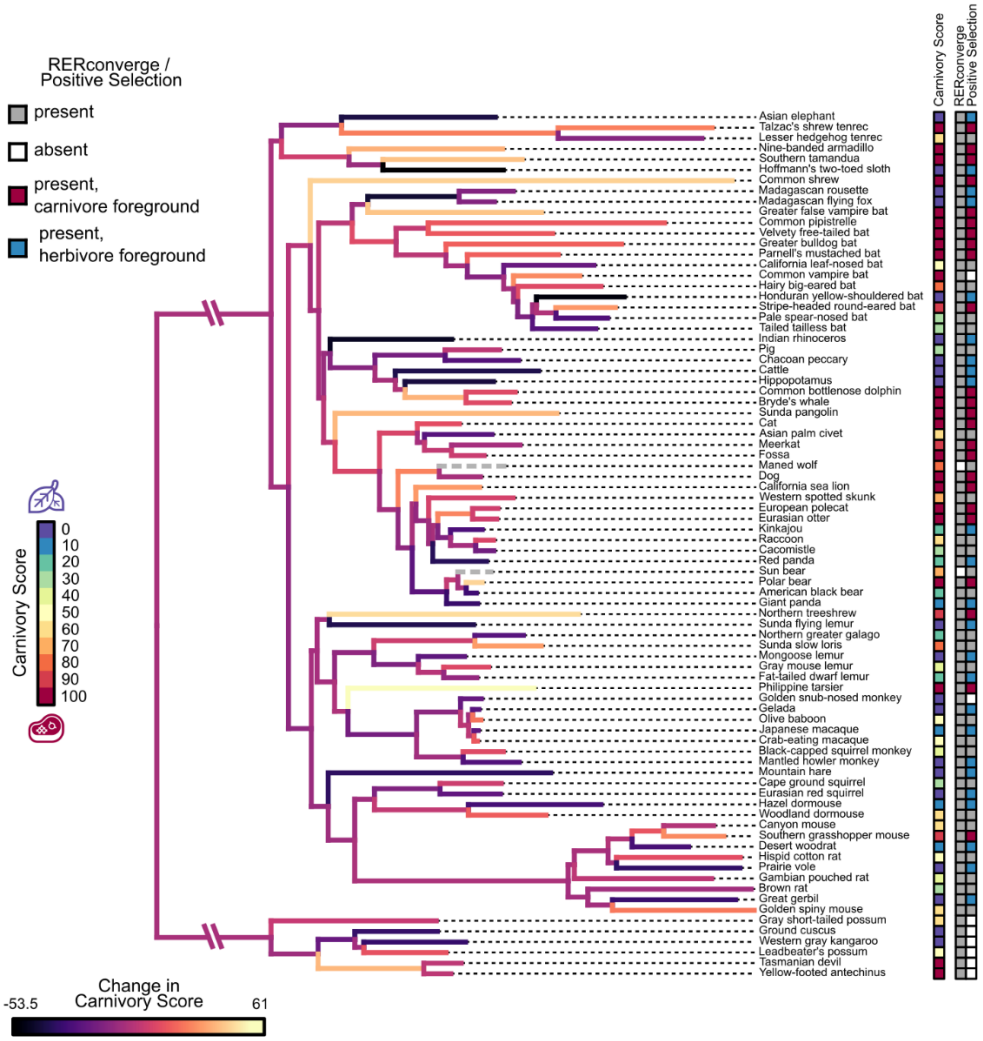

Figure 1. Species selection for comparative genomic analyses of mammalian diet. The carnivory score represents the proportion of the diet composed of animal-based food items for each species, as listed in EltonTraits (17). This score was used as input in our RERconverge analyses and informed selection of foreground species in our tests for positive selection. Branch colors represent the magnitude and direction of change in carnivory score across the phylogeny, as inferred by using fast estimation of maximum likelihood ancestral states (20). Bright yellow and dark purple branches indicate increases and decreases in carnivory score, respectively. Gray dashed branches represent species that were not included in our RERconverge analyses and so were not used to estimate change in carnivory score. Branch length represents the average evolutionary rate across all genes for a given branch, as reported by RERconverge. Most species were included in both the RERconverge and positive selection analyses and are marked as present (gray, red, blue) in the corresponding columns. Species not included in an analysis are marked as absent (white). For the positive selection analyses, species included in the carnivorous (carnivory score ≥90) and herbivorous (carnivory score ≤10) foregrounds are marked in the corresponding column as red and blue, respectively. Scientific names and carnivory scores are provided for each species in Additional File 1: Table S1.

Associating relative evolutionary rates with the evolution of diet

We used RERconverge in R (21) to identify genes with consistent changes in evolutionary rate associated with the evolution of the quantitative carnivory score. RERs reveal deviation in evolutionary rate along a specific branch of the phylogeny, based on genome-wide expectations. A highly positive RER may indicate positive selection or relaxation of constraint. A highly negative RER indicates that increased constraint has led to fewer substitutions than expected. RERconverge estimates the correlation between RER and change in a phenotype across a phylogeny. A significant negative correlation between RER and change in carnivory score corresponds to slower evolution among lineages evolving towards increased carnivory, and faster evolution in lineages leading to less carnivorous species. Increased evolutionary constraint implies that the gene contributes more to fitness under a particular phenotypic condition, so we focused our analyses on genes with significant negative correlations when identifying genes associated with increasing carnivory (22). Conversely, for genes related to increasing herbivory, we looked at genes with significant positive correlations.

We used the *estimatePhangornTreesAll* wrapper function from RERconverge, which utilizes a maximum likelihood approach implemented in phangorn (23), to calculate branch lengths for each gene while fixing the topology of the gene trees to match the maximum clade credibility phylogeny of Upham et al. (24). These trees were used as input for the RERconverge analyses alongside our carnivory scores. We winsorized the RERs of each gene so that the three most extreme values at each end were set to that of the next most extreme RER. This minimized the influence of outliers. We implemented the default branch length filtering in RERconverge, setting the shortest 5% of branches across all gene trees to N/A. This resulted in an additional 1,205 genes being filtered for having fewer than 60 branches remaining in the tree, leaving 13,912 remaining genes in this analysis.

To evaluate confidence in gene-trait associations inferred by RERconverge, we used a phylogenetically restricted permutation strategy (‘permulations’; 22, 25). We randomly simulated carnivory scores for each species 100,000 times using a Brownian motion model of evolution. Then for each simulated set of values, the observed carnivory scores were assigned to species based on the ranks of the simulated scores. We used these shuffled phenotype sets to calculate gene-trait associations using RERconverge, generating 100,000 null statistics per gene. We

calculated a new, empirical *P*-value for each gene-trait association based on the proportion of null statistics that are equally or more extreme than the observed statistic for that association. We corrected for multiple hypothesis testing using Storey's correction method (26; FDR=0.05). Using the Mann-Whitney U test for enrichment available in RERconverge, we identified biological functions associated with the evolution of diet. We constructed tissue-specific gene sets from the Genotype-Tissue Expression Project (GTEx; 51) data, defining tissue-specificity following the strategy of Jain and Tuteja (27). We tested for tissue-specific enrichment using these gene sets, gene pathway enrichment using KEGG and Reactome pathways from MSigDB (28), and enrichment for abnormal phenotypes using gene sets from the Mammalian Phenotype (MP) Ontology (29).

Positive selection associated with the evolution of carnivory or herbivory

We tested each gene for positive selection via branch-site models (branch-site neutral versus branch-site selection) using codeml from PAML (30), as implemented in BASE (31). We also used BUSTED from the HyPhy software suite (32, 33). These models test for positive selection in a group of foreground species chosen *a priori*. Terminal branches leading to species with carnivory scores ≥90 (n=25) and ≤10 (n=24) were selected as foreground for carnivory- and herbivory-specific analyses, respectively (Fig. 1; Additional File 1: Table S1). Select species with carnivory scores of 20—specifically the red panda and kinkajou—were included in the herbivore foreground despite relatively high carnivory scores because they are considered in the broader literature to be primarily herbivorous (34-38). Nucleotide alignments for each gene were obtained from Zoonomia (6, 7).

PAML branch-site models do not test for the absence of positive selection in background species, so these tests alone do not identify genes experiencing positive selection in association with a trait of interest (39). To confirm that our significant branch-site results corresponded to foreground-specific positive selection, we removed the foreground species and tested for tree-wide positive selection using site models (M1a versus M2a) in PAML. Only genes detected as positively selected by the branch-site model and not the site model with foreground species excluded were retained (Additional File 2: Fig. S1).

We employed a conservative strategy to generate final lists of positively selected genes. To account for the impact of gene tree discordance on our results (40), we performed positive selection

analyses using gene trees and the fixed species tree. We used RAxML v8.2.12 (41) with the GTRGAMMAI nucleotide substitution model to generate gene trees. We only considered a gene to have experienced diet-associated positive selection when it produced a significant result in both our codeml and BUSTED analyses, and if this result persisted when using the gene tree and the species tree (Additional File 2: Fig. S1). As an additional filtering step, we checked that the signals of positive selection returned by codeml and BUSTED were not caused by relaxation of selection by using RELAX (42), and excluded any genes showing significant relaxation from our final gene lists (Additional File 2: Fig. S1). We used overrepresentation analyses to test our final lists for enrichment of GO terms, gene pathways from the Reactome and KEGG databases, and phenotypes from the MP Ontology using clusterProfiler and ReactomePA (43, 44). For all overrepresentation analyses, we used human annotation databases.

RESULTS

Molecular evolutionary constraint associated with dietary change

We identified genes that experienced convergent evolutionary constraint or positive selection in association with changes in carnivory across a phylogeny of 80 mammal species. Of the 13,912 genes included in our RERconverge analysis, we identified six with a significant negative correlation between relative evolutionary rate (RER) and change in carnivory score (FDR=0.05; Table 1; Fig. 2A; Additional File 1: Table S3). These significant correlations are a result of convergent rate changes in multiple regions of the phylogeny (Additional File 2: Fig. S2). A negative correlation indicates that these genes evolved slower, implying that they make a greater contribution to fitness the larger the increase in carnivory score (22). Among genes with this signature, the products of three (*SLC13A2*, *SLC14A2*, and *CLDN16*) are involved in transport or resorption in the kidney, two (*CPB1* and *PNLIP*) are secreted pancreatic enzymes, and one (*ACADSB*) is involved in amino acid metabolism (45-50). Our analysis had greater power to detect negative associations between RERs and change in carnivory score compared to positive associations. The results of a Mann-Whitney U test indicated a significant difference in the distribution of empirical, permulation-based *P*-values for genes with positive and negative correlations (AUC=0.523, $P=2.16 \times 10^{-6}$), with fewer low *P*-values generated for positively correlated genes (Additional File 2: Fig. S3).

Table 1. Genes with evolutionary rates that are significantly associated with change in carnivory score. Permulation *P*-values represent the proportion of 100,000 permulations that produced a stronger correlation with change in carnivory score than the observed value for each gene. Multiple hypothesis testing corrections were performed by generating *Q*-values using Storey's correction method (26; FDR=0.05). A negative correlation (Rho) indicates that the gene evolves slower and may be of greater functional importance in lineages with larger positive changes in carnivory score.

Gene	Rho	Parametric *P*-value	Parametric *Q*-value	Permulation *P*-value	Permulation *Q*-value
SLC14A2	-0.446	1.54×10^{-8}	2.14×10^{-4}	$<1.00 \times 10^{-5}$*	<0.035*
SLC13A2	-0.358	6.94×10^{-6}	0.037	$<1.00 \times 10^{-5}$*	<0.035*
CLDN16	-0.400	7.93×10^{-6}	0.037	1.00×10^{-5}	0.035
CPB1	-0.325	8.02×10^{-5}	0.150	1.00×10^{-5}	0.035
PNLIP	-0.350	1.85×10^{-5}	0.065	2.00×10^{-5}	0.046
ACADSB	-0.306	3.36×10^{-4}	0.260	2.00×10^{-5}	0.046

* After generating 100,000 null statistics, none produced a stronger correlation with diet than the observed values for *SLC13A2* and *SLC14A2*. However, if the *P*-values are adjusted to the smallest observable non-zero *P*-value (1.00×10^{-5}), they would produce a significant empirical *Q*-value (FDR=0.05).

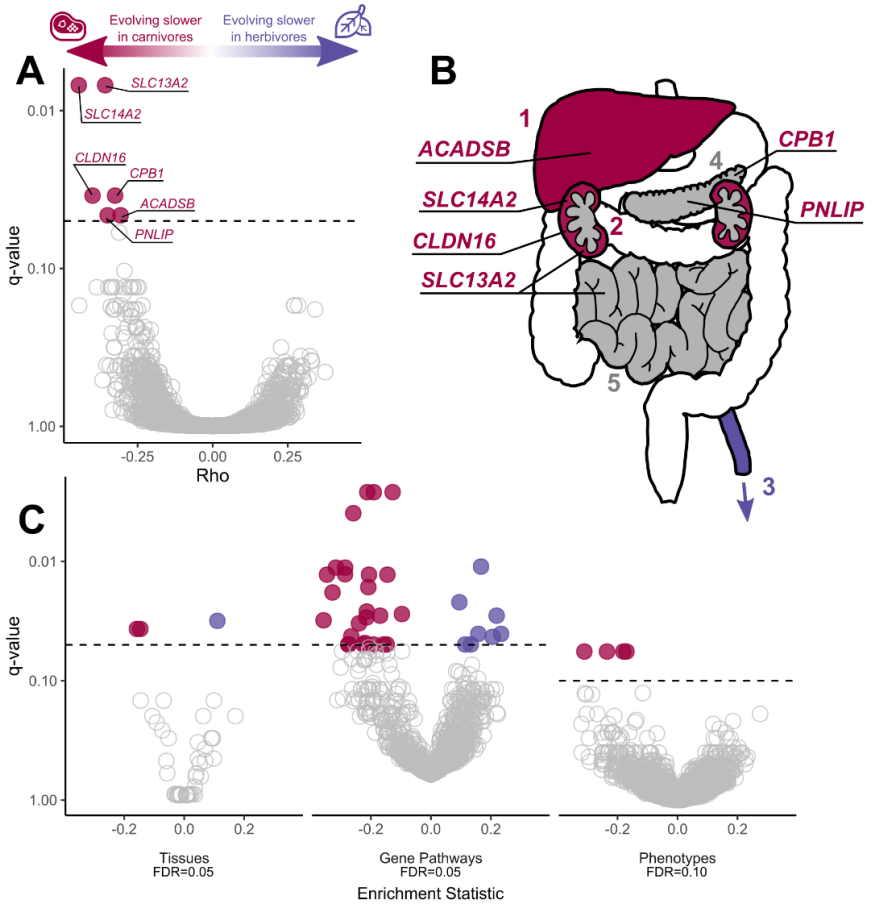

Figure 2. Top genes and pathways with signatures of diet-associated evolutionary constraint. (*A*) Genes identified by RERconverge as having a significant association between relative evolutionary rate and change in carnivory score. A negative correlation (Rho) indicates that the gene evolves slower, and may be of greater functional importance, the larger the increase in carnivory score. A positive correlation indicates slower evolution with larger decreases in carnivory score. After 100,000 permulations, six genes showed a significant association, and each evolved slower in association with increased carnivory (FDR=0.05). (*B*) Tissues associated with positive and negative correlations, where: 1 = liver, 2 = kidneys, 3 = tibial artery, 4 = pancreas, 5 = small intestine. Some tissues are annotated with the genes found to be individually significant. The annotated tissues are the locations of highest expression for those genes in adult humans, according to the Genotype-Tissue Expression Project (51). The gene sets of red organs were more evolutionarily constrained in lineages with increasing carnivory. Blue indicates more evolutionary constraint in lineages with decreasing carnivory. Gray represents organs that were not significantly enriched but were sites of strongest expression for slowly evolving genes. (*C*) Biological functions showing constraint associated with change in diet. We used RERconverge to test for enrichment of gene sets representing tissues (n=50), gene pathways (n=1,290), and abnormal phenotypes (n=3,560). For tissues, two gene sets were enriched for negative correlations, and one set was enriched for positive correlations. For gene pathways, 26 and 8 gene sets were enriched for negative and positive correlations, respectively. For abnormal phenotypes, four gene sets were enriched for negative correlations.

Each of the six significant genes are most strongly expressed in the liver, kidneys, pancreas, or small intestine (51). We performed a gene set enrichment analysis to determine if there was a statistically significant association between change in carnivory score and the rate of evolution of genes expressed in specific tissues. Our test showed that the liver and kidney cortex gene sets were significantly enriched and correlated with slower evolution in lineages with positive changes in carnivory score (FDR=0.05; Fig. 2B,C; Table 2; Additional File 1: S4). In contrast, the tibial artery was significantly enriched for genes evolving more slowly in lineages with negative changes in carnivory score.

Table 2. Tissues that are significantly enriched for genes associated with change in carnivory score. Permulation *P*-values represent the proportion of 100,000 permulations that produced a stronger enrichment test statistic than the observed value for each gene set. Multiple hypothesis testing corrections were performed by generating *Q*-values using Storey's correction method (26; FDR=0.05). A negative enrichment statistic indicates that the gene set is enriched for genes evolving slower with increasing carnivory scores.

Tissue	Enrichment Statistic	Parametric *P*-value	Parametric *Q*-value	Permulation *P*-value	Permulation *Q*-value
Tibial Artery	0.111	2.64×10^{-4}	0.002	6.30×10^{-4}	0.032
Liver	-0.158	1.28×10^{-19}	6.38×10^{-18}	0.002	0.037
Kidney Cortex	-0.146	7.42×10^{-7}	1.24×10^{-5}	0.002	0.037

We extended our enrichment analysis to 1,290 gene pathways from the Reactome and KEGG databases and identified significant enrichment for 34 pathways (FDR=0.05; Fig. 2C; Additional File 1: Table S5). Of these pathways, 26 were associated with slower evolution in lineages with larger increases in carnivory. These pathways had functions related to digestion, biological oxidation, transport of small molecules, heme degradation, and metabolism of amino acids, lipids, and carbohydrates (Fig. 3). Eight pathways evolved at slower rates in lineages with decreases in carnivory score. These pathways were associated with disparate functions: cardiovascular disease, cell motility, signaling by anaplastic lymphoma kinase (ALK) in cancer, and signaling by Hippo, NOTCH3, and interleukin-17 (Additional File 2: Fig. S4).

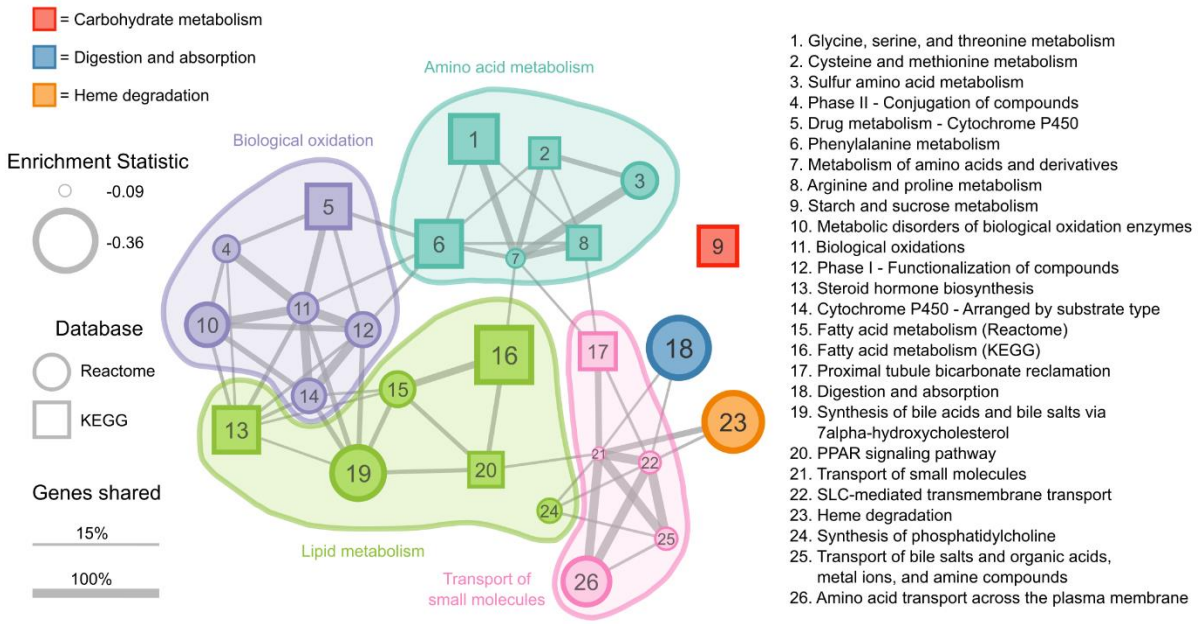

Figure 3. Significantly enriched gene pathways (n=26) under greater evolutionary constraint in lineages with increasing carnivory scores. Each circle or square represents a gene pathway. Circles and squares represent pathways from the Reactome and KEGG databases, respectively. The size of the shape represents the magnitude of the difference between the distribution of test statistics for genes in that pathway and the distribution for all other genes. Larger shapes, representing pathways enriched for genes with more negative correlation statistics, indicate pathways that experience greater constraint as carnivory score increases. The width of lines connecting pathways represents the proportion of shared genes in the smaller gene set. Colors represent the broad functional categories that the pathways occupy.

We performed an enrichment analysis on 3,560 gene sets representing abnormal phenotypes from the Mammalian Phenotype Ontology (29). We identified four phenotypes with significant enrichment using both raw and permulation-based empirical *P*-values (FDR=0.1; Fig. 2C; Table 3; Additional File 1: Table S6). All four gene sets were enriched for genes evolving slower as carnivory score increases. Two phenotypes—decreased urine osmolality (MP:0002988) and increased urine calcium level (MP:0005441)—were related to abnormal urine homeostasis, and the other two—abnormal lipid level (MGI:0001547) and abnormal circulating lipid level (MGI:0003949)—were associated with lipid homeostasis. The abnormal urine homeostasis gene sets shared 8 of 82 total genes, with 4—*SLC12A1*, *KCTD1*, *SLC4A1*, and *UMOD*—in the 10 top-ranked genes for both gene sets. The abnormal lipid homeostasis gene sets shared 10 of 134 total genes, with 1 overlapping gene, *BHMT*, in the top 10 for both sets.

Table 3. Phenotype-based gene sets from the Mammalian Phenotype Ontology that are significantly enriched for genes associated with carnivory score. Permulation *P*-values represent the proportion of 100,000 permulations that produced a stronger enrichment statistic than the observed value for each gene set. Multiple hypothesis testing corrections were performed by generating *Q*-values using Storey's correction method (26; FDR=0.1). A negative enrichment statistic indicates that the gene set is enriched for genes evolving slower in lineages with increasing carnivory scores.

Phenotype	Enrichment Statistic	Parametric *P*-value	Parametric *Q*-value	Permulation *P*-value	Permulation *Q*-value
MP:0003949 abnormal circulating lipid level	-0.235	3.30×10^{-5}	0.011	7.00×10^{-5}	0.057
MP:0001547 abnormal lipid level	-0.170	3.62×10^{-9}	1.20×10^{-5}	8.00×10^{-5}	0.057
MP:0005441 increased urine calcium level	-0.311	7.74×10^{-8}	8.53×10^{-5}	8.00×10^{-5}	0.057
MP:0002988 decreased urine osmolality	-0.181	1.01×10^{-5}	0.006	8.00×10^{-5}	0.057

Positive selection

We identified 193 and 172 genes that showed significant positive selection in the most carnivorous and herbivorous species, respectively (Additional File 1: Tables S7, S8). Among the carnivore-specific positively selected genes, we identified enrichment for four Reactome pathways related to *O*-linked glycosylation and one pathway related to pregnenolone biosynthesis (Table 4). The ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) genes driving the observed enrichment for *O*-linked glycosylation pathways were also components of the only GO Term that was significantly enriched—metallopeptidase activity (GO:0008237; Table 4). We did not identify significant enrichment of any phenotypes, gene pathways, or GO Terms in our list of positively selected genes in herbivores.

Table 4. Gene sets enriched for positive selection in the most carnivorous mammals (carnivory score ≥90). We did not detect enrichment in the most herbivorous mammals (carnivory score ≤10).

Gene Set	# Genes analyzed	Positively selected genes	P-value	Q-value
		Reactome Pathways		
O-linked glycosylation	87	*ADAMTS5*, *ADAMTS12*, *ADAMTS14*, *ADAMTS20*, *B4GALT6*, *CHST4*, *LARGE2*, *THSD4*	1.83×10^{-5}	0.008
Defective B3GALTL causes Peters-plus syndrome (PpS)	34	*ADAMTS5*, *ADAMTS12*, *ADAMTS14*, *ADAMTS20*, *THSD4*	7.70×10^{-5}	0.013
O-glycosylation of TSR domain-containing proteins	35	*ADAMTS5*, *ADAMTS12*, *ADAMTS14*, *ADAMTS20*, *THSD4*	8.89×10^{-5}	0.013
Diseases associated with *O*-glycosylation of proteins	49	*ADAMTS5*, *ADAMTS12*, *ADAMTS14*, *ADAMTS20*, *THSD4*	4.50×10^{-4}	0.041
Pregnenolone biosynthesis	12	*CYP11A1*, *FDX2*, *STARD3NL*	4.58×10^{-4}	0.041
		GO Terms		
GO:0008237 metallopeptidase activity	158	*ADAMTS5*, *ADAMTS12*, *ADAMTS14*, *ADAMTS20*, *AEBP1*, *CNDP1*, *CPZ*, *PEPD*, *STAMBPL1*, *THSD4*, *TRHDE*	5.75×10^{-6}	0.002

DISCUSSION

We identified genes that are functionally important during mammalian adaptation to increasingly carnivorous or herbivorous diets using quantitative carnivory scores and a phylogeny of 80 species sampled from the taxonomic and dietary breadth of Mammalia. Ours is the first comparative genomic study of diet to use a quantitative variable and to analyze convergence across so many species. We consistently found stronger signals of convergent genic evolution accompanying increasing levels of carnivory compared to decreasing carnivory (Table 1; Additional File 1: Tables S7, S8). Convergence in constraint correlated with increasing carnivory appears to act on lipid and amino acid metabolism, biological oxidation, and liver and kidney functions (Fig. 2; Tables 2-4; Additional File 1: Tables S5, S6).

The roles of liver and kidneys in adaptation to carnivory

Multiple lines of evidence support that liver and kidney functions are crucial for the convergent evolution of increased carnivory. Of the six genes identified by RERconverge as evolving significantly slower with increasing carnivory scores, three (*SLC13A2*, *SLC14A2*, and *CLDN16*) and one (*ACADSB*) are respectively most strongly expressed in human kidneys and liver (51). Further, the kidney cortex and liver gene sets were enriched for genes showing increased evolutionary constraint in correlation with increasing carnivory scores (Table 2).

The importance of kidney function to carnivory may be mediated by its role in the reabsorption and secretion of small molecules. Our enrichment analysis identified several pathways related to small molecule transport, including several containing solute carrier proteins (SLCs) such as SLC13A2 and SLC14A2, that were under greater constraint the more a lineage increased in carnivory. Many of these pathways take place in the kidneys. The results of our phenotype-specific enrichment analysis also support a central role of small molecule transportation in the carnivore kidney. Of the four MGI phenotype gene sets that were significantly enriched, two of them—decreased urine osmolality (MP:0002988) and increased urine calcium level (MP:0005441)—are related to abnormal urine homeostasis, which is a direct consequence of impaired renal function. Mammals excrete nitrogenous waste in the form of urea, and the urea load that must be excreted each day largely depends on the protein content of an organism's diet. The most carnivorous species have the greatest urea loads and produce urine with higher urea concentrations than other species (52). The need to excrete greater quantities of nitrogenous waste imposes selective pressure on the carnivore kidney. This is supported by our finding that *SLC14A2* is evolutionarily constrained in carnivorous lineages, as the urea transporters encoded by this gene are critical to the urea-dependent urine concentrating mechanism (46).

Liver function is important during adaptation to carnivory due to its role in amino acid and lipid metabolism. It is the only organ in mammals that can completely metabolize most amino acids, and it plays an active role in amino acid synthesis (53). Carnivorous diets are rich in proteins and lipids. Pathways related to their metabolism should therefore be under stronger purifying selection in carnivores, as lipids and amino acids represent their main energy sources. Among genes strongly constrained with increasing carnivory, we identified enrichment for genes involved in several processes related to the metabolism of lipids and assorted amino acids, including many that take

place within the liver. One of these pathways was related to bile acid synthesis. Bile acids facilitate digestion of dietary fats and allow excess cholesterol to be excreted from the body. Indeed, bile acid synthesis and excretion represent the major mechanism of cholesterol catabolism and elimination in mammals (54). Given that carnivores possess lipid-rich diets, lipid metabolism pathways may be evolutionarily constrained so that sufficient levels of cholesterol homeostasis are maintained in these species.

Metabolism of amino acids and lipids is associated with biological oxidation, and we found that several biological oxidation pathways evolved slower in lineages with increasing carnivory scores. Thus, it is an integral component of carnivory. However, increased constraint on biological oxidation in lineages with increasing levels of carnivory is an unexpected result, given the importance of xenobiotic metabolism in herbivores: Many plants employ chemical defenses against herbivory (55), and herbivores may overcome these defenses via oxidation (56, 57). A negative correlation between the RERs of biological oxidation genes and carnivory score may be driven by rapid evolution in increasingly herbivorous lineages. However, we do not detect these genes in the results of our herbivore-specific positive selection analyses.

Amino acid and lipid metabolism outside of the liver are also associated with adaptation to increasing carnivory. The evolutionary rates of *CPB1* and *PNLIP*—two genes most strongly expressed in the pancreas (51)—were significantly negatively correlated with increasing carnivory scores. The gene for another pancreatic lipase, *PNLIPRP2*, had a marginally significant association with carnivory score (Rho=-0.311; Q=0.059) and may be important to carnivory. Both lipases have been implicated in the dietary switch to carnivory during the evolution of Cetacea (9, 12). Our results support previous findings that pancreatic lipases play a role during evolutionary changes in degree of carnivory. Interestingly, another member of this family of pancreatic lipase genes, *PNLIPRP1*, has convergently lost function in multiple herbivorous mammals, suggesting a relaxation of selective constraint in herbivores compared to carnivores (10). The protein encoded by this gene, PL-RP1, shows little to no detectable lipase activity and instead acts as a competitive inhibitor of pancreatic lipase (58). Inactivation of *PNLIPRP1* would therefore enhance fat digestion capacity in herbivores. However, we observed no significant association between *PNLIPRP1* and carnivory score, likely because the gene alignments used in our study do not include pseudogenes.

Biological oxidation outside the liver is important for dietary adaptation: our positive selection analyses showed that a member of the cytochrome P450 gene superfamily, *CYP11A1*, is positively selected in the most carnivorous species. The protein encoded by *CYP11A1* catalyzes the first stage of steroid hormone synthesis—the production of pregnenolone from cholesterol via oxidation (59). The pregnenolone biosynthesis pathway was enriched in our carnivore-specific positive selection analysis (Table 4), and steroid hormone synthesis showed increased constraint in lineages with increasing carnivory scores in our analysis of gene evolutionary rates (Additional File 1: Table S5).

In summary, our results indicate that, during adaptation to more carnivorous diets, purifying selection acts on functions related to metabolism and elimination of substances found in excess in animal-based foods. The liver and kidneys have been disproportionately targeted during carnivorous adaptation to maintain sufficient functioning of these processes.

Implications of carnivorous adaptations for human health

Our results offer insights into diet-related diseases in humans and suggest avenues for new medical research. Human dietary maladaptation is not driven by high rates of carnivory *per se*: stable isotopes and archeological evidence indicate that increased meat consumption began early in human evolution (60, 61) and that pre-agricultural diets were more carnivorous than current ones (62). Instead, an evolutionarily rapid shift away from pre-agricultural diets in favor of fattier meats and processed foods has increased chronic disease in modern communities (63). Our findings help characterize mechanisms of human dietary maladaptation because metabolic challenges posed by extreme carnivory overlap with those posed by modern processed diets. For example, carnivore adaptations that allow for lipid-rich diets without disease are medically relevant given a global increase in the consumption of high-fat foods in human populations (64). In humans, high intakes of red meat are associated with increased blood cholesterol levels and coronary heart disease (65). Tightly regulated cholesterol and lipid homeostasis may be an adaptation to avoid such diseases in carnivores. Our phenotype-specific enrichment analysis supports that lipid homeostasis is a main feature of successful adaptation to increasing carnivory. Two overlapping significantly enriched phenotype gene sets—abnormal lipid level (MGI:0001547) and abnormal circulating lipid level (MGI:0003949)—are related to lipid homeostasis and showed greater importance in

lineages with increasing carnivory scores. Thus, the lipid metabolism genes driving our results may be important targets for future study.

Evolutionary constraint on the carnivore kidney is relevant to human health due to the elevated burden of high-protein diets on renal function. High-protein diets have been linked to kidney damage and reduced renal function in observational human studies (66). In other omnivorous mammals, experimental evidence has shown that high protein intake causes kidney inflammation and damage (67, 68). Protein-rich diets have been implicated in kidney stone formation as they increase the excretion of calcium and oxalate into the urine (69). The link between diet and kidney stones offers a plausible connection between *SLC13A2*, carnivory, and human health, as defects in this gene cause low urinary citrate concentrations, leading to the formation of kidney stones (70). The kidney stone connection also strengthens the link for *CLDN16* because defects in this gene are associated with elevated urine calcium levels and deposition of calcium salts in the kidneys (71).

Another constrained pathway in lineages with increasing carnivory is heme degradation. While nonheme iron is present in both plant- and animal-based foods, heme iron is mainly found in animal tissues (72). Primarily carnivorous species ingest excess heme, so heme degradation is a more important process in these species. Excess heme intake causes oxidative damage, inflammation, and cell death (73), and has been implicated in several diseases, including type-2 diabetes, coronary heart disease, gut dysbiosis, colitis, and cancers (74-76). Cancer risk is highest among carnivorous mammals (77), and selection for DNA repair mechanisms in carnivores has been attributed to increased consumption of heme-related reactive oxygen species (11). Increased disease risk due to heme consumption may drive the increased selective constraint acting on heme degradation in lineages with increasing levels of carnivory.

Carnivore-specific positive selection acting on *O*-linked glycosylation pathways and metalloprotease activity may have relevance to human diabetes. Enrichment for these pathways was primarily driven by four *ADAMTS* genes. *O*-linked glycosylation is a widespread post-translational modification of ADAMTS metalloproteases (78). The positively selected ADAMTSs are associated with extracellular matrix (ECM) assembly and degradation in various biological processes (79-84). In mice, high-fat diets and insulin resistance increase expression of ECM remodeling genes and encourage collagen deposition, leading to fibrosis in tissues such as skeletal

muscle, liver, and adipose tissue (85-87). High-fat diets and healthy glucose metabolism that resembles diabetes (2) may increase selective pressure acting on ECM-regulating proteases in carnivores.

Comprehensive sampling elucidated fundamentally important functions

Our analyses sampled the taxonomic and dietary breadth of Mammalia, incorporating genome-wide data from 80 species. The signals of convergent molecular evolution that we detected are driven by multiple instances of dietary convergence across the phylogeny (Additional File 2: Fig. S2). We therefore isolated genes and biological functions that are important for carnivory and herbivory in most or all instances that such diets evolved across mammals. Some of our results overlap with those previously found in studies of fewer species. For example, lipid metabolism has frequently been identified as a target of selection in carnivores (e.g., 3, 9, 11). However, our study is the first to emphasize the fundamental importance of kidney and liver function during the convergent evolution of carnivory.

By including more species in our analyses, we provide a clearer picture of which diet-specific selection signatures are clade-specific and which are apparent in all carnivores. For example, Kim et al. (11) found carnivore-specific selection acting on functions such as neuron development, muscle strength, and agility. The authors attribute their findings to agile hunting behaviors. However, we did not detect this selection in our broader analysis. Some carnivores in our expanded sampling are not agile predators and are unlikely to have experienced selection on these functions. For example, Bryde's whale was included in our analyses and feeds on zooplankton with limited movement capabilities (88). Bryde's whale does not require the same agility while hunting as the carnivorans, orca, and Tasmanian devil that were analyzed by Kim et al. (11).

Herbivores showed less signal of convergent genic evolution

We obtained fewer significant results when focusing on selective pressures accompanying increased herbivory. Our RERconverge analysis did not identify any genes that evolved significantly slower in association with decreasing carnivory scores, and only eight gene pathways were enriched for genes with greater functional importance as herbivory increases. These pathways were related to cell motility, immunity, cardiovascular disease, and signaling by Hippo, NOTCH3, and ALK (Additional File 1: Table S5). We may have identified fewer signals of molecular constraint in lineages with increased herbivory because of a difference in power to detect positive

and negative associations between RER and carnivory score. We had reduced power to detect positive correlations, which represent increasing constraint as carnivory score decreases (Additional File 2: Fig. S3). Thus, fewer significant results for increases in herbivory may not have a biological explanation. Alternatively, the weaker signal of molecular constraint may indicate that genic adaptation is less predictable in response to increased herbivory than in response to increased carnivory.

The relevance to diet or health of pathways constrained in lineages with decreasing carnivory scores is generally less obvious than those with the opposite pattern of constraint. These pathways may represent understudied components of dietary adaptation. For example, Hippo signaling integrates signals from multiple sources to regulate cell proliferation and differentiation, and can influence and be influenced by the metabolism of glucose, lipids, and amino acids (89). Given that multiple metabolic cues are integrated into Hippo signaling, it is unclear why constraint strengthens with increasing levels of herbivory. NOTCH3 plays an important role during vascular development and in continued vascular functioning of adult organisms (90-92). Given this, enrichment for NOTCH3 signaling may be related to enrichment of the tibial artery gene set in the same direction (Table 2). However, it is unclear why vascular function would be under stronger evolutionary constraint as herbivory increases. ALK activation is involved in the development of many cancers (93), but its function beyond cancer is not as well understood. It is thought to play a role in nervous system development (94), but expression has also been detected in the small intestine and colon (95). More recently, ALK signaling was implicated in regulating energy expenditure and weight gain (96), so it might play an important, unidentified role in diet regulation, and further study could elucidate its relevance.

Despite limited signal and unclear relevance for many results associated with increasing herbivory, one pathway stood out. The importance of interleukin-17 (IL-17) signaling in lineages adapting to more herbivorous diets may reflect IL-17's role in gut microbiome regulation. IL-17 encourages the production of antimicrobial proteins and the migration of neutrophils into infected intestinal mucosa, maintains intestinal barrier integrity, and limits gut dysbiosis (97-100). Gut microbiomes are essential for herbivores. The plants consumed by herbivores comprise complex polysaccharides that cannot be digested by mammalian enzymes. However, microorganisms in the herbivore gut can ferment these compounds to produce metabolites that are used easily by their

host. The importance of microorganisms for plant consumption has led to convergence in gut microbiota across mammalian herbivores, while those of carnivores are highly variable (101, 102). As gut microbes are necessary for successful herbivory, increasing selective constraint should act on gut homeostasis pathways in lineages becoming more herbivorous.

CONCLUSION

An understanding of the genomic mechanisms underlying mammalian dietary adaptation offers insights for improving human health. In this work, we analyzed 80 species to identify genes that experienced convergent molecular evolution during evolutionary changes in degree of carnivory. Six genes—*ACADSB*, *CLDN16*, *CPB1*, *PNLIP*, *SLC13A2*, and *SLC14A2*—experienced convergent molecular constraint in lineages that became more carnivorous. During the evolution of increasing carnivory, purifying selection acted on liver and kidney functions to maintain metabolism and excretion of substances found in excess in animal-based diets. Some of the metabolic challenges imposed by extreme carnivory mirror those presented by dietary maladaptation to modern human diets. Thus, our findings present new avenues for research of diet-related disease.

SUPPLEMENTARY INFORMATION

File name: **Additional File 1**
File format: Excel spreadsheets (.xlsx)
Title: Table S1
Description: Species selection for comparative genomic analyses of mammalian diet.

Title: Table S2
Description: GO Term overrepresentation in genes that were filtered from downstream analyses (n=2322).

Title: Table S3
Description: Gene-phenotype evolutionary rate correlation.

Title: Table S4
Description: Tissue enrichment analysis results from RERconverge.

Title: Table S5
Description: Gene pathway enrichment analysis results from RERconverge.

Title: Table S6
Description: Phenotype enrichment analysis results from RERconverge.

Title: Table S7
Description: Genes experiencing positive selection in the most carnivorous mammals (carnivory scores ≥90).

Title: Table S8
Description: Genes experiencing positive selection in the most herbivorous mammals (carnivory scores ≤10).

File name: **Additional File 2**
File format: Word document (.docx)
Title: Figure S1
Description: Conservative strategy for generating final lists of positively selected genes associated with carnivory and herbivory in mammals.

Title: Figure S2
Description: Changes in relative evolutionary rates (RERs) across the mammalian phylogeny for genes significantly correlated with change in carnivory score.

Title: Figure S3
Description: Significant differences in the distribution of permulation *P*-values for positive and negative associations between gene relative evolutionary rates (RERs) and carnivory score

Title: Figure S4
Description: Significantly enriched gene pathways (n=8) under greater evolutionary constraint in lineages with decreasing carnivory scores.

DECLARATIONS

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
Nucleotide and amino acid alignments used in this study were generated by the Zoonomia Consortium (https://zoonomiaproject.org/the-data/). Carnivory scores for species included in this study are available in Additional File 1. Our RERconverge and positive selection results are available in Additional File 1. Code generated for this project is available on GitHub (https://github.com/mdpllard/carnivory-genes). The tissue- and mouse phenotype-specific gene sets generated for our enrichment analyses are available on GitHub.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
MDP and WKM performed data pre-processing. MDP analyzed the data and interpreted results with EEP and WKM. MDP wrote the manuscript. EEP and WKM edited the manuscript. EEP supervised the study.

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