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**Running head (50 characters)**: *Trpc2* infers loss, not gain, of bat vomerolfaction

**Title**: *Trpc2* Pseudogenization Dynamics in Bats Reveal Ancestral Vomeronasal Signaling, then Pervasive Loss

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**Abstract**

Comparative methods are often used to infer loss or gain of complex phenotypes, but few studies take advantage of genes tightly linked with complex traits to test for shifts in the strength of selection. In mammals vomerolfaction detects chemical cues mediating many social and reproductive behaviors and is highly conserved, but all bats exhibit degraded vomeronasal structures with the exception of two families (Phyllostomidae and Miniopteridae). These families either regained vomerolfaction after ancestral loss, or there were many independent losses after diversification from an ancestor with functional vomerolfaction. In this study, we use the *Transient receptor potential cation channel 2* (*Trpc2*) as a molecular marker for testing the evolutionary mechanisms of loss and gain of the mammalian vomeronasal system. We sequenced *Trpc2* exon 2 in over 100 bat species across 17 of 20 chiropteran families. Most families showed independent pseudogenizing mutations in *Trpc2*, but the reading frame was highly conserved in phyllostomids and miniopterids. Phylogeny-based simulations suggest loss of function occurred after bat families diverged, and purifying selection in two families has persisted since bats shared a common ancestor. As most bats still display



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pheromone-mediated behavior, they might detect pheromones through the main olfactory system without using the *Trpc2* signaling mechanism.

Discovering the evolutionary trajectory and selective forces shaping complex traits across the Tree of Life is a central task of evolutionary biology. The intricate mechanisms underlying organismal complexity inspired Louis Dollo's evolutionary "law" in 1893 (Dollo 1893). Dollo describes evolution as "irreversible" and "discontinuous", and trait complexity prevents evolution from returning organisms to their previous state. But phylogenies and comparative methods to infer complex trait evolution have challenged Dollo's law (Collin and Cipriani 2003; Domes et al. 2007; Lynch and Wagner 2010; Wiens 2011). These studies infer the re-evolution of traits after ancestral loss, sometimes multiple times. Those analyses remain controversial, however, as unsuitable inference methods have been used, and the true complexity of traits is often ignored (Goldberg and Igić 2008). Few studies have actually turned to the molecular mechanisms determining trait function (Sassi et al. 2007; Christin and Besnard 2009; Seher et al. 2012), or the selective forces shaping traits. Here, we analyze the molecular evolution of a mammalian chemosensory system, examining changes in selection underlying the loss of this complex system throughout the diversification of bats.

Chemical signaling and perception among conspecifics in mammals occurs via pheromones and other chemical cues detected in the secondary olfactory system by a sense known as vomerolfaction (Fig. 1). Pheromones play an important role in social communication and mediate many behaviors critical to fitness, including conspecific recognition, courtship and mating, parental care, territoriality, and even the detection of sick individuals (Fortes-Marco et al. 2013; Boillat et al. 2015; Martín-Sánchez et al. 2015). Pheromones are often highly specific, and are key regulators of offspring and group recognition in mammals (Smadja and Butlin 2009; Pitcher et al. 2011, 2015; Rizvanovic et al. 2013; Cinková and Policht 2014; Péron et al. 2014). With a few exceptions, vomerolfaction is ubiquitous across mammals.



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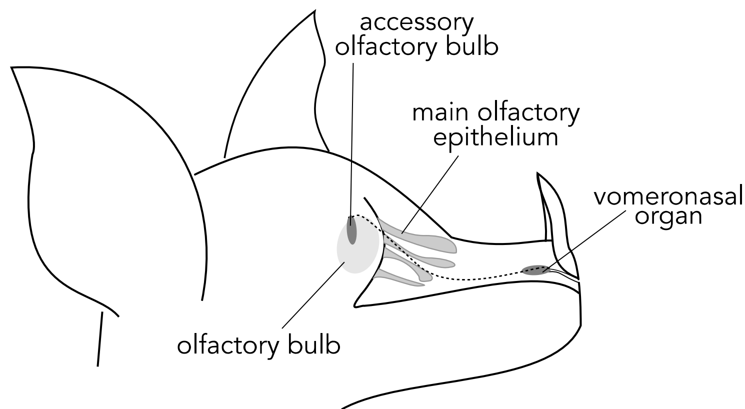


**Fig 1.** Schematic of a sagittal interior view of a bat nose depicting the two nasal chemosensory systems in mammals. The main olfactory system primarily innervates the olfactory bulb, while the vomeronasal organ primarily innervates the accessory olfactory bulb (dotted line).

Different environments and resources may select for fine-tuned perception of one particular sense over another. Because the neural pathways of sensory systems are energetically expensive to maintain (Niven and Laughlin 2008), selection on senses no longer critical to fitness may relax, and sensory organs may become vestigial (Fong et al. 1995). Well-known examples include blindness in cavefish (Jeffery et al. 2003; Jeffery 2005), and loss of sweet taste in carnivores (Jiang et al. 2012). Because of its importance to survival and reproduction, mammalian vomerolfaction has been lost in only a few groups, and in most such cases these losses are associated with changes in ecology. For example, independent losses in cetaceans, sirenians, and some pinnipeds probably relate to relaxed selection for nasal chemosensory perception under water (Yu et al. 2010; Kishida et al. 2015). In contrast, losses in Old World primates have been explained by the switch to a diurnal niche, presumably following greater reliance on visual cues (Garrett et al. 2013).

The loss of vomerolfaction in bats is poorly understood. Most bats exhibit highly degraded or absent vomeronasal organs (Fig. 2; Table S1) and nonfunctional genes for detecting pheromones (Cooper and Bhatnagar 1976; Frahm and Bhatnagar 1980; Wible and Bhatnagar 1996; Bhatnagar and Meisami 1998; Bhatnagar and Smith 2007; Young et al. 2010). This loss is puzzling because bats are



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nocturnal, and often live in colonies of many individuals and exhibit a diversity of social systems of the kind requiring pheromone communication. Based on these observations, one hypothesis might link the evolution of flight and echolocation to reduced selective pressure on a well-developed social smell system, generating a potential shift between primary sensory modalities in bats. There are, however, two major exceptions to bat vomeronasal loss: New World Leaf-nosed bats (Phyllostomidae) and Long-fingered bats (Miniopteridae). Species in these families have well developed vomeronasal machinery (Wible and Bhatnagar 1996; Bhatnagar and Meisami 1998; Bhatnagar and Smith 2007; Zhao et al. 2011). Exactly how this pattern has evolved remains poorly understood, and the selective forces shaping this system are unclear.

In mammalian vomerolfaction, the *Transient receptor potential cation channel, subfamily C, member 2* (*Trpc2*) gene is an indicator of a functioning vomeronasal system, as it is seemingly indispensable for pheromone signal transduction (Mast et al. 2010). Trpc2 is a transmembrane ion channel that becomes activated after a pheromone binds to the G-protein-coupled vomeronasal receptors in the neurons of the vomeronasal organ (Liman and Dulac 2007). This signal is primarily relayed to the accessory olfactory bulb in the brain (Fig. 1), where it is interpreted (Keverne 1999). The disruption of numerous social behaviors through loss of Trpc2 signaling has been well established (Yu 2015). In rodents, *Trpc2* knockout males mount both males and females, while knockout females mount as if they were males and reduce their maternal care for pups because they cannot detect and process the pheromonal cues that normally mediate this behavior (Leypold et al. 2002; Stowers et al. 2002; Kimchi et al. 2007; Hasen and Gammie 2009; Yu 2015). A functional *Trpc2* is tightly linked to a functioning mammalian pheromone-signaling pathway, providing an ideal molecular marker for modeling vomeronasal evolution.

Pseudogenes are the genetic signatures of once-functional systems on which selection has relaxed. Mammalian lineages lacking vomeronasal structures frequently have a pseudogenized *Trpc2*, indicating a sense no longer under negative selection. Multiple aquatic and amphibious mammals, Old



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World primates, and twelve species of bats, have pseudogenized *Trpc2* genes (Zhang and Webb 2003; Yu et al. 2010; Zhao et al. 2011). A previous study noted only three of the 15 analyzed species of bats with an intact *Trpc2*, including two phyllostomids and one miniopterid (Zhao et al. 2011). The ratio of rates of nonsynonymous to synonymous substitutions (ω) can be used to characterize selection (or its absence) on a gene, with neutrally evolving genes having equal rates of amino acid changing and silent substitutions (ω=1). The shift from strong purifying selection on functional genes (ω<<1) to relaxed selective pressure will gradually shift ω closer to 1, so that genes on which selection has relaxed more recently will display lower ω than genes pseudogenized for longer. Although some mammalian *Trpc2* studies quantify ω, noting lower ω in branches with lineages with an intact *Trpc2*, most focus on the presence and absence of disruptive mutations. Here, we use the molecular evolution of vomeronasal signaling mechanisms to explicitly model alternative evolutionary hypotheses, and discover the evolutionary dynamics leading to the present-day pattern of function and loss in the vomeronasal system.

We propose two evolutionary scenarios for vomeronasal loss in bats. First, and more parsimonious based on the distribution of vomeronasal system loss (see Fig. 1 for morphology; Fig. S1 for parsimony reconstruction), the ancestor of all bats lost vomeronasal function, with two instances of secondary gain of the system in two families of bats (Wible and Bhatnagar 1996). If so, *Trpc2* reactivated after ancestral selection had relaxed, perhaps to the point of inactivation. In the second, and less parsimonious scenario, *Trpc2* has remained under strong purifying selection throughout the early diversification of bats, and has more recently lost function at least six different times (Zhao et al. 2011). Given sufficient sampling and appropriate models, it is possible to distinguish between these two scenarios by estimating the rates of synonymous and amino acid-changing substitutions of internal branches of the phylogeny (Sassi et al. 2007).

To distinguish between these hypotheses, and illuminate the evolutionary forces shaping vomeronasal evolution in bats, we modeled the evolution of *Trpc2* with a large taxonomic sample



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across nearly all families of bats. Using rates of molecular evolution estimated throughout the phylogeny, we simulated ancestral sequences to model the probability of the two alternative evolutionary histories of the bat vomeronasal system. Our findings demonstrate how models of molecular evolution can inform the mechanisms shaping complex trait gain or loss in a comparative framework. Besides illuminating the evolutionary mechanisms of loss of a seemingly indispensable sense for perceiving and responding to chemically mediated social behaviors, our analyses illuminate a sensory system whose function continues to puzzle biologists.

**Materials and Methods**

Trpc2 *Amplification*

To model evolutionary changes in vomeronasal signal transduction in bats, we generated new *Trpc2* sequences for 104 species (Fig. 2, Table S2), representing an order-of-magnitude gain in taxonomic sampling over the only previous study (Zhao et al. 2011). We focused most of our sampling on families in which *Trpc2* was hypothesized to be functional: Miniopteridae (n=7) and Phyllostomidae (n=85). Phyllostomids are particularly species rich, with >170 lineages, and we sampled ~50% of known species. Using a previously published primer pair for each species (Zhao et al. 2011), we amplified the second exon of the *Trpc2 β*-isoform (~496 bp), the form of the protein primarily expressed in the vomeronasal organ (Yu et al. 2010). This is the longest exon of the 13-exon gene (~900 amino acids in the entirety of Trpc2) for this isoform, and encodes for an ankyrin-repeat domain associated with protein-protein interaction (Yu et al. 2010). We obtained bat tissue samples from several museum collections (Table S2 for museum accession numbers). We used the following PCR protocol for each 25-μL reaction: 12.5μL Econotaq Plus Green (Lucigen), 3μL 10μM forward primer, 3μL 10μM reverse primer, 2-4μL template DNA, and 2.5-4.5μL $dH_2O$. The following protocol was used for amplification: 94˚C for 5 min; 35 cycles of 94˚C 30s denaturation, ~58˚C 30s annealing, and 72˚C extension; and 72˚C 5 min final extension. Annealing temperatures were adjusted for different species, but all protocols had an annealing temperature between 52-60˚C. Amplicons



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were sequenced using standard Sanger sequencing methods provided by the University of Arizona Genetics Core Facility. All sequences were manually checked for quality using Geneious version 8.1.7 (Kearse et al. 2012).

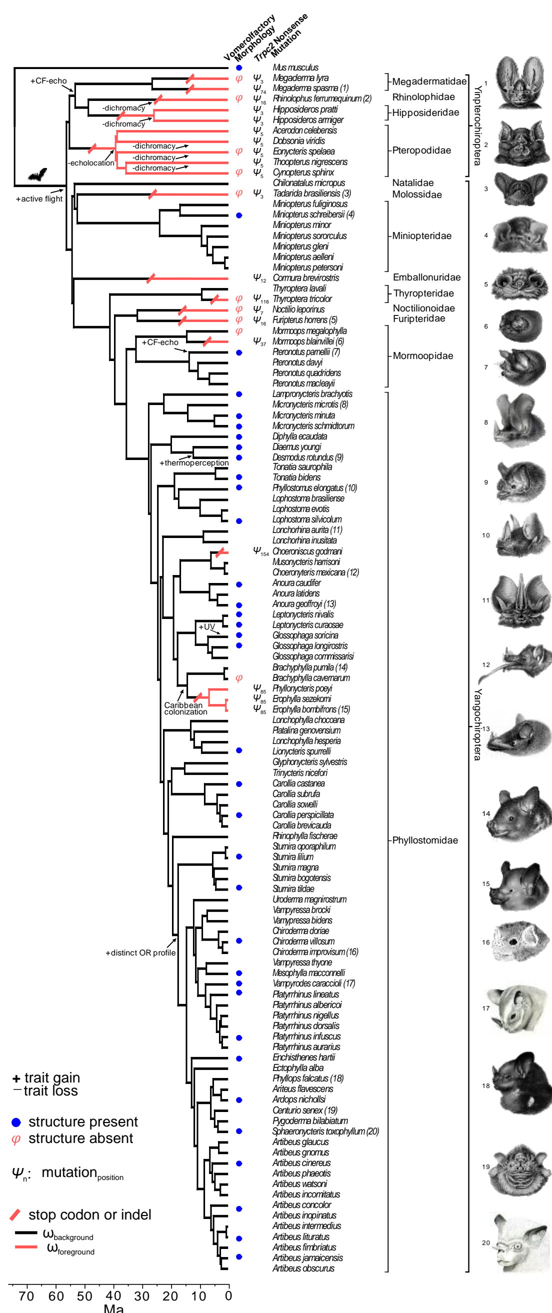


**Fig. 2**. Patterns of pseudogenization in *Trpc2* exon 2 in bats sampled. Red slashes indicate either stop codon or indel mutation that rendered the protein nonfunctional. Some mutations were shared among



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lineages. Black branches were used to calculate the background rates ($\omega_{background}$) for PAML and RELAX tests. Branches marked in red are lineages with pseudogenized *Trpc2* experiencing relaxed selection and were used as foreground rates ($\omega_{foreground}$). Nonsense mutations occur first in the reading frame unless the mutation is shared, for which the first shared mutation in the reading frame is listed (Figure S2 for full description). Suborders and families are labeled. See Table S1 for references for morphological characters. Arrows to the respective node or branch indicate either environmental shifts, or sensory specializations or losses (Table S3 for references). Dichromacy is the ability to detect two distinct color wavelengths and all species, including *Mus musculus* are dichromatic unless otherwise noted. Image number designates the corresponding lineage and illustration. Illustration credit: Ernst Haeckel, public domain (1, 2, 5, 11, 19); Alcide d'Orbigny, public domain (3); G. H. Ford, public domain (4); Adrián Tejedor, under license (6, 7, 8, 9, 13, 14, 15, 18); George E. Dobson, public domain (10, 12); Nicholas Holowka, with permission (16); Bonnie Sumner, with permission (17, 20).

For comparative analyses, we combined new sequences from 103 species with previously published bat *Trpc2* exon 2 sequences (13 species), and the *Trpc2* exon 2 for *Mus musculus* (Zhao et al. 2011). Two species (*Pteropus vampyrus* and *Myotis lucifugus*) present in a previous analysis (Zhao et al. 2011) were not included here, as published sequences were from a different exon, unavailable from published bat genomes. All sequences were aligned using the Geneious aligner. The alignment of previously published sequences was visually inspected and manually corrected to ensure our alignments matched those from Zhao et al. (2011). To identify nonsense and indel mutations, we used both the open reading frame of *Mus musculus* and the reading frame presented in Zhao et al. (2011) as proxies for the functional exon. *Trpc2* was considered a pseudogene if a stop codon and/or frameshift mutation was present in the sequence.

*Analyses of Molecular Evolution*



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Early in pseudogene evolution, single base pair substitutions accumulate (Marshall et al. 1994). In the absence of constraints, amino-acid changing substitutions occur at a higher rate than when the protein was under purifying selection (Marshall et al. 1994; Sassi et al. 2007). Eventually, more dramatic disruptive mutations, such as nonsense mutations or indels shifting the codon reading frame, may fix (Marshall et al. 1994). Pseudogenes have likely evolved neutrally for many generations, accumulating nonsynonymous changes as quickly as synonymous substitutions before stop codons or indels become fixed. In the continued absence of selection, the formerly functional gene will eventually become a set of nucleotides impossible to relate to the protein it once encoded.

If a nonsense mutation or indel is present, *Trpc2* may be evolving neutrally. In contrast, an intact gene reflects purifying selection, although in some cases the open reading frame may have evolved neutrally in the recent past. We tested for purifying and relaxed selection by estimating the ratio of rates of nonsynonymous to synonymous mutations ($\omega$), in which $\omega$ close to 0 indicates purifying selection, and $\omega$ nearer to 1 suggests relaxed selection. To estimate these rates, we used two maximum likelihood methods implemented in PAML and RELAX (Yang 2007; Wertheim et al. 2014). PAML estimates $\omega$ for different branch classes and RELAX distinguishes if $\omega$ estimates correspond to relaxed or strong selection. Both programs required a phylogeny. We inferred the *Trpc2* gene tree using maximum-likelihood (see Supplemental Methods), but this tree was incongruent with established species trees, likely because of the short exon reading frame. Therefore, we used a used a published phylogeny of phyllostomids (Rojas et al. 2016), and combined it with a published dated phylogeny of all bats (Shi and Rabosky 2015). The tree was pruned to match the data using the *geiger* package in R (Harmon et al. 2008). Branch lengths were ignored, as PAML and RELAX estimate these. Both programs also required the codon structure to be maintained, with no stop codon mutations in the reading frame. Thus, we removed nonsense sites by converting stop codons to gaps and removing the entire column from insertion mutations. Deletions were kept intact to maintain the reading frame. Downstream sequences after these mutations were kept in order to maximize the



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amount of information in the alignment. After removing and editing these sites, 163 codons were available for nearly all lineages. We expected higher $\omega$ in pseudogenized branches compared to lineages with an intact gene.

To model molecular evolution across bats, we first set up a null model estimating a single $\omega$ across all branches (M0) in the PAML v. 4.8e *codeml* routine (Bielawski and Yang 2005; Yang 2007). We then tested two models in which branches were binned into distinct classes. First, we classified the tree into two distinct classes (M2.1): those with nonsense and indel mutations (test/foreground branches) and those retaining an intact *Trpc2* exon, including internal branches on the tree (background branches). Our second test classified the tree into three distinct classes (M2.2): those with nonsense and indel mutations (same test branches as M2.1), those terminal branches for lineages with an intact reading frame, and all internal branches on the tree. We then fitted the *codeml* M2 branch model to estimate $\omega$ for the background branches ($\omega_{background}$) and test ($\omega_{foreground}$) branches for M2.1 (Bielawski and Yang 2004). For M2.2, we fit the M2 branch model to estimate $\omega$ for the background branches ($\omega_{background}$), and two test branch classes ($\omega_1$ for functional foreground branches, $\omega_2$ for nonfunctional foreground branches). To determine which model better fit the data, we used a likelihood ratio test of M2 against the null M0.

We also used the program RELAX implemented in HyPhy v. 2.22 to test for two distinct rates of $\omega$ between lineages with pseudogenizing mutations versus all other branches, and to distinguish positive from relaxed selection because increased $\omega$ may be indicative of either (Pond et al. 2005; Wertheim et al. 2014). RELAX first estimates $\omega$ among three rate classes for each branch using a branch site-random effects likelihood (BS-REL) method and then fits a parameter $k$ indicating the strength of selection. $\omega$ rate classes are transformed by raising to the power of $k$ ($\omega^k$), such that $k > 1$ pushes high and low classes away from one (indicative of strong selection) or $k < 1$ scales the high and low rate classes towards one (indicative of relaxed selection). We estimated a single $k$ ($k = 1$) for



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all branches and then fitted a model estimating *k* for each of the two branch classes specified in the PAML analyses.

*Simulation Experiments*

We used simulations to validate our ω estimates. Using the *evolverNSbranches* routine in PAML, we simulated 1,000 sets of codon alignments modeled under the same evolutionary parameters as the *Trpc2* data for all simulated scenarios. To test the robustness of the observed ω estimates, we expanded on the framework of a previous study that used codon models to test for relaxed selection (Sassi et al. 2007). First, to evaluate the false positive rate, we simulated alignments under a null model centered on the estimated M0 value of ω. The simulated codon alignments matched the number of codons and number of species of the alignment, and the estimated branch lengths, transition/transversion ratio, and codon frequencies of M0. We then applied M2.1 branch models in *codeml* to each replicate dataset to obtain a distribution of $\omega_{background}$ and $\omega_{foreground}$. This analysis tested whether values recovered from the data are consistent with a null process of evolution at a single ω throughout the tree.

Second, to distinguish between alternative hypotheses of vomeronasal evolution, we modeled two scenarios of *Trpc2* evolution. If *Trpc2* were functional in the ancestor of bats and was subsequently pseudogenized on multiple independent branches, then ω estimated for the background branches of the tree would reflect strong purifying selection, and would be distinctly lower than estimates for pseudogenized foreground branches. Hence, values estimated from the data should fit within non-overlapping distributions for background ($\omega_{background}$) and foreground branches ($\omega_{foreground}$) simulated ω from the M2.1 model (Hypothesis A, Fig. 3).

Alternatively, if *Trpc2* had been reactivated twice after evolving neutrally as bats first evolved, then *Trpc2* background branches would reflect these high ω values, until the two "reactivation" events on the ancestral branches of the families Phyllostomidae and Miniopteridae. We



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simulated background and foreground branches with increased ω values (ω = 0.5). If the evolutionary history of *Trpc2* were consistent with this scenario, then frequency distributions of background and foreground ω should overlap (Hypothesis B, Fig. 3), and values estimated from the data should fall within the distributions of this simulation. For all simulations, we tested whether resulting distributions were distinct using a two-sample Student's *t* test.

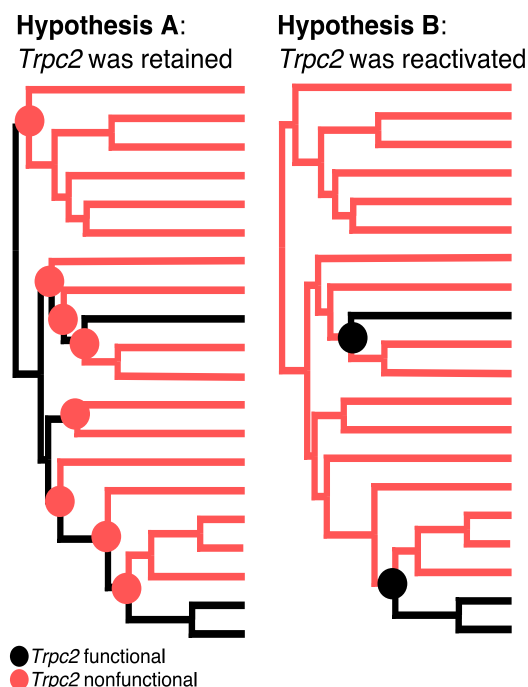


**Fig. 3**. Hypothesized evolutionary scenarios of *Trpc2* evolution.

## Results

To estimate the evolutionary history of vomerolfaction in bats, we sequenced the second exon of *Trpc2* of more than 100 species of bats, including every chiropteran super family and three quarters of the 20 families in the order (see Fig. 2, Table S2 for species sampled). The alignment spanned 163 codon positions, approximately 20% of the 891-amino acid protein described in the mouse (Yu et al. 2010), and included all sites analyzed by Zhao et al. (2011). We detected 13 independent pseudogenizing mutations in *Trpc2* across bats (Table S4; Fig. 2), twice the number found in a previous study (Zhao et al. 2011), with several species containing multiple pseudogenizing mutations



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within the sequence (Table S4; Fig. S2). New sequencing revealed intact genes in all surveyed members of the family Miniopteridae (n=7), and in a majority of surveyed members of Phyllostomidae (n=81). Surprisingly, however, two instances of *Trpc2* pseudogenization were recorded within phyllostomids: a small monophyletic clade of three Caribbean endemic nectar-feeding bats shared a single base pair deletion at codon position 88 (Table S4, Fig. S2), demonstrating a recent pseudogenization in the ancestor of these three species, and a premature stop codon at codon position 154 in the nectarivorous phyllostomid *Choeroniscus minor* (Table S4, Fig. 2, S1). Wider taxonomic sampling also revealed pseudogenization events in six other families, all within the suborder Yangochiroptera. The ratio of the rates of nonsynonymous to synonymous substitutions was used to model molecular evolution in *Trpc2*. We tested whether separate estimates of ω for nonfunctional lineages and functional/internal branches (M2) were a better model fit than a single ω for all branches in PAML. All models were tested with five different initial values for ω, and all parameter estimates converged on the same values. M2.1 and M2.2 provided significantly better fit than the null model (Table 1; M2.1: $\chi^2_{(1)} = 157.2$; $p = 1.0$ E-6; M2.2: $\chi^2_{(2)} = 157.2$; $p = 1.0$ E-6), and we were able to estimate two distinct ω values for two branch classes. The two-branch class model (M2.1) and the three-branch (M2.2) class model had identical likelihoods. Thus, we used M2.1 for all subsequent analyses, as it was the best-fit model with the fewest parameters. Non-functional species had ω five times higher ($\omega_{foreground} = 0.541$) than the background ω of species with functional *Trpc2* ($\omega_{background} = 0.096$), indicating relaxation of negative selection. As stop codon and frameshift triplets were removed from the alignment to run the routines, the estimates of $\omega_{foreground}$ severely underestimate ω, and thus provide a lower limit to the true value of ω for these branches.

**Table 1**. Estimated parameters of relaxed selection tests using branch models in PAML.

| Model | $\omega_{background}$ | $\omega_{foreground}$ | κ | TL | np | lnL | LR | *p* |
|---|---|---|---|---|---|---|---|---|
| *M0 (null)*: one ratio model | 0.216 | 0.216 | 6.76 | 6.72 | 231 | -6054 | --- | --- |



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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *M2.1:* two branch classes | 0.096 | 0.541 | 6.80 | 6.71 | 232 | -5975 | 157.2 | <<0.001 |
| *M2.2:* three branch classes | 0.085 | $\omega_1$: 0.541<br>$\omega_2$: 0.101 | 6.80 | 6.71 | 233 | -5975 | 157.2 | <<0.001 |

ω: ratio of nonsynonymous to synonymous substitutions; $\omega_{background}$ for M0 is ω for all branches, for M2 ω for internal and functional branches for the M2: two branch class model; $\omega_1$: foreground for nonfunctional branches; $\omega_2$: foreground for functional branches: transition/transversion rate; TL: tree length; np: number of parameters; lnL: log-likelihood; LR: likelihood ratio; *p*: *p*-value of likelihood ratio test of M2 models relative to the null M0 model. Grey indicates the best-fit model used for interpretation and simulations.

The variation in ω associated with pseudogenization was corroborated with RELAX, which estimates the ω statistic for every branch on the tree and a parameter *k* to indicate varying strength of selection. The null model estimates ω for each branch of the tree but does not transform the branches, while the alternative model estimates a value of *k* that transforms ω for two specified branch classes and tests if the alternative better fits the data. The RELAX model with a *k* parameter transforming ω across the tree for two branch classes, fit significantly better than the null (Table 2; $\chi^2_{(1)} = 124.8$; $p = 1.0E{-6}$). This indicates selection in lineages with nonfunctional *Trpc2* relaxed relative to the background and functional branches, as *k* is much less than 1 ($k = 0.10$).

**Table 2**. Estimated parameters of relaxed selection using RELAX. $k = 1$ is the null hypothesis. $k > 1$ indicates positive selection and $k < 1$ indicates relaxed selection.

| **Model** | **lnL** | **np** | **AIC$_c$** | **TL** | ***k*** | **LR** | ***p*** |
|---|---|---|---|---|---|---|---|
| $\omega_{background}$ v. $\omega_{foreground}$ | | | | | | | |
| null | -5941 | 254 | 12397 | 6.50 | 1 | --- | --- |
| alternative | -5878 | 255 | 12273 | 6.54 | 0.10 | 124.8 | <<0.001 |

*lnL*: log-likelihood; *np*: number of parameters; *AICc*: sample sized corrected Akaike



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Information Criterion; *TL*: tree length; *k*: selection intensity; LR: likelihood ratio; *p*: *p*-value of likelihood ratio of alternative relative to null for each test

The ω estimates from the simulated M2.1 data did not overlap with any background ($t$ = -144.8, degrees of freedom [df] = 1471, $p$ = 2.2E-16) and foreground ($t$ = 132.5, df = 1381, $p$ = 2.2E-16) estimates from the null model simulations (Fig. 4). This implies low Type I error and that our estimates of ω from the best-fit M2.1 model are robust.

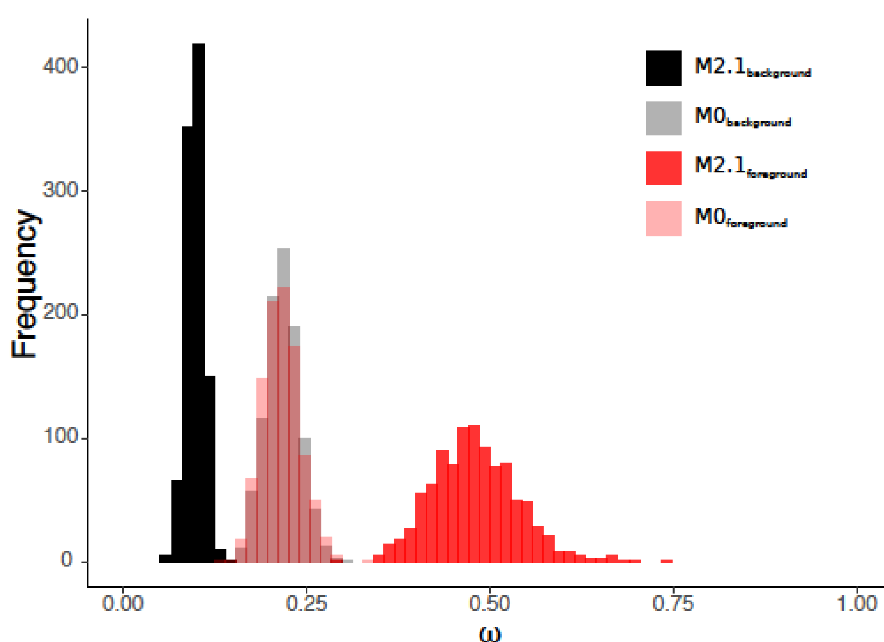


**Fig. 4**. Background and foreground ω estimates of null and alternative codeml simulations.

Figure 5 displays results of the simulated evolutionary scenarios modeled using the M2.1 branch classes (Fig. 5A), in which *Trpc2* had experienced purifying selection along internal branches (Fig. 5B: $\bar{x}_{\text{background}}$ = 0.097; 95% confidence interval [CI]: 0.096, 0.097; $\bar{x}_{\text{foreground}}$ = 0.479; CI: 0.477, 0.482) or selection had relaxed throughout internal branches of the tree (Fig. 5C: $\bar{x}_{\text{background}}$ = 0.545; CI: 0.544, 0.546; $\bar{x}_{\text{foreground}}$ = 0.546; CI: 0.542, 0.550). The values of $\omega_{\text{background}}$ and $\omega_{\text{foreground}}$ estimated



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from the data (Table 1) fall well within the respective simulated distributions in Hypothesis A and are completely inconsistent with Hypothesis B (Fig. 3). This strongly suggests *Trpc2* function has been independently lost and was functional during early bat diversification.

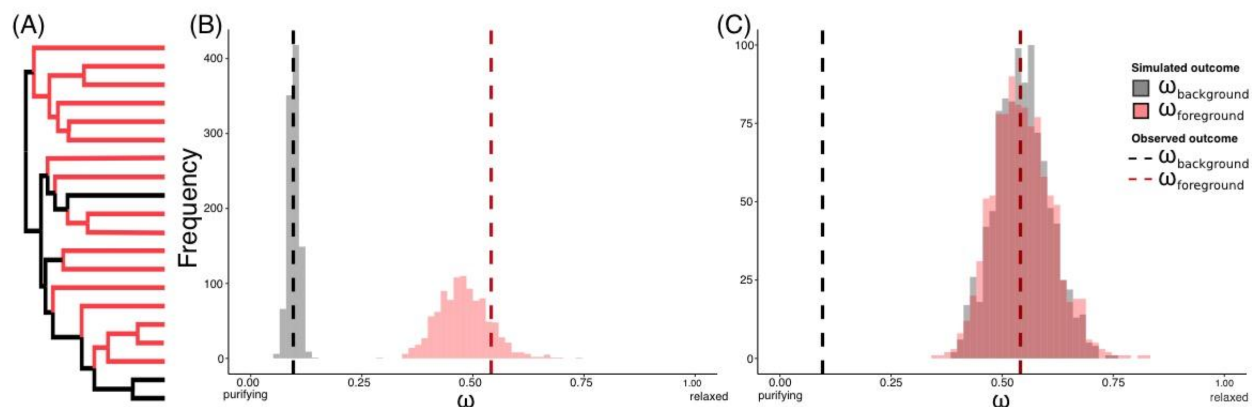


**Fig. 5**. (A) Background (black) and foreground (pink) branches from the M2.1 used in the simulation experiment. (B) Simulated and estimated ratios of nonsynonymous to synonymous substitutions (ω) in *Trpc2*. If *Trpc2* had been under strong purifying selection, simulated distributions of functional lineages and internal branches ($\omega_{background}$) should be distinct from simulated distributions of estimates of nonfunctional lineages ($\omega_{foreground}$) and the null model. (C) If *Trpc2* had been reactivated, $\omega_{background}$ estimates should overlap with $\omega_{foreground}$. Dotted lines indicate values of ω estimated from the data.

**Discussion**

Pheromone chemosensation mediated by the vomeronasal system has rarely been lost in terrestrial mammals (Yu et al. 2010). Hence, the widespread pseudogenization of *Trpc2* in bats is surprising, and counter to expectations based on trait-based analyses of vomeronasal function and loss. We sought to characterize the forces shaping the evolution of *Trpc2* and vomerolfaction in bats by modeling molecular evolution to test whether selection relaxed prior to, or following the diversification of major extant bat families. Understanding the sequence of shifts in selection provides evidence for explanations for relaxed selection, such as a invading a new environment or sensory exchange. We discovered that the evolutionary history of vomerolfaction in bats is more complex than



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previously appreciated. We outline three key findings: [1] complete pseudogenization of *Trpc2* has independently evolved at least 13 times across bats, including twice within one family in which most species have functional vomeronasal organs; [2] while relaxed selection explains these multiple losses of function, at least two large clades have retained *Trpc2* under strong purifying selection; and [3] once *Trpc2* function was lost, it was never regained. Unlike in aquatic mammals or mammals switching to primarily visual communication, the pattern of vomerolfactory losses in bats seems to be decoupled from any obvious ecological explanation. While the explanation for vomerolfactory loss in bats remains mysterious, our findings also highlight the power of molecular evolution to inform the evolution of complex phenotypes across phylogenies.

Trpc2 *loss in mammals*

In nearly all mammals, pheromone-mediated behavior is critical to survival and reproduction. From maternal aggression when protecting young from intruders to sex identification during courtship and mating (Stowers et al. 2002; Kimchi et al. 2007; Liberles 2014; Corona and Lévy 2015), and from territorial urine-marking to reproductive synchronization (Rasmussen and Schulte 1998; Hurst and Beynon 2004; Stockley et al. 2013), the machinery to detect and respond to pheromones is conserved and under strong selection from the platypus to platyrrhine (New World) primates. Severe disruptions in social behavior occur when *Trpc2* is rendered non-functional, such as failing to care for offspring, or mistaking males from females, and vice versa (Liman and Dulac 2007; Hasen and Gammie 2009; Fraser and Shah 2014; Yu 2015). Unsurprisingly, this gene is highly conserved. In fact, among nonvolant mammals, widespread sampling has revealed just four independent cases of pseudogenization of the *Trpc2* gene: in the ancestor of the fin whale (*Balaenoptera physalus*) and dolphin (*Tursiops truncatus*), in the river otter (*Lutra lutra*), in the harbor seal (*Phoca vitulina*), and in the ancestor of catarrhine primates (Zhang and Webb 2003; Yu et al. 2010; Ibarra-Soria et al. 2014). In bats, we found more than three times as many losses of function as in all non-flying mammals. While extant bats have evolved 13 independent nonsense mutations in *Trpc2* since they last shared a



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common ancestor ~60 Ma (Shi and Rabosky 2015), similar disruptions have evolved only four times in the ~150 Ma divergence among all other modern mammals (dos Reis et al. 2012).

Mapping the absence of vomeronasal structures across bat families supports ancestral organ loss followed by a small number of system gains (Wible and Bhatnagar 1996). *Trpc2* may have been evolving neutrally before selection strengthened it in the ancestors of families possessing a functional gene today. While reactivation of a pseudogene is uncommon, it is not impossible (Marshall et al. 1994; Takuno et al. 2008). A pseudogene with a frameshift or stop codon mutation could be repaired by back-mutation, a previously deleted segment by an insertion from a closely related gene, exon shuffling, or gene conversion (Doxiadis et al. 2006; Takuno et al. 2008).

Alternatively, strong purifying selection on *Trpc2* could have persisted during diversification of all major bat lineages, and subsequently relaxed independently in multiple bat families. Losses of function in critical vomeronasal signal transduction have been independent, with the first loss occurring ~50 Ma or before (Table S4, Fig. S2). Estimates of $\omega$ for internal branches and functional lineages ($\omega_{background}$) were much lower than those from lineages with detectable loss-of-function mutations ($\omega_{foreground}$), indicating selection has relaxed in many branches. The $\omega$ values estimated from the data were completely inconsistent with simulations with constant low $\omega$ (uniform strongly negative selection) or higher $\omega$ (uniform relaxed selection), and were consistent simulations of distinct $\omega$ background and foreground branches. Together, these results support strong purifying selection on *Trpc2* has persisted in both internal branches and extant functional lineages. Additionally, the narrow distribution and low value of $\omega_{background}$ indicates selection did not relax in those branches, while the higher value and greater variance of $\omega_{foreground}$ reflects the varying age of pseudogenization events across the phylogeny (Fig. 5B): recent pseudogenization events have not accumulated as many mutations as older events. Instead of reactivation, quantitative analyses of evolutionary rates support the retention of functional *Trpc2* in two bat families.

*Changes in environment did not influence* Trpc2 *loss in bats*



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Change in environment is one of two main explanations of vomeronasal loss and pseudogenization of *Trpc2* among non-volant mammals (Zhang and Webb 2003; Liman 2006; McGowen et al. 2014; Kishida et al. 2015). For aquatic mammals, a shift to life underwater made it impossible to disperse and detect pheromonal cues in a manner similar to terrestrial mammals. Cetaceans and sirenians have highly degraded olfactory and vomeronasal morphology; dolphins and whales show ancestral pseudogenization of *Trpc2*, and carnivoran harbor seals and sea otters have pseudogenized *Trpc2* (Mackay-Sim et al. 1985; Yu et al. 2010; McGowen et al. 2014). Bats are the only mammals capable of powered flight, but this shift seems unrelated to vomeronasal loss. Airborne pheromonal cues and chemical cues transmitted during roosting interactions are still effective in the aerial environment of bats. Bats also possess a diversity of external glands, many of them sexually dimorphic, that excrete scented compounds, suggesting chemical cues are widely used (Scully et al. 2000). Finally, the evolution of flight in the ancestor of extant bats is decoupled from the multiple independent pseudogenization events detected (Fig. 2).

A shift from nocturnal to diurnal habits has also been suggested to change selective pressure on vomerolfaction. Though not all diurnal primates lack vomerolfaction, all nocturnal primates have functional vomeronasal systems (Evans and Schilling 1995; Bhatnagar and Meisami 1998; Smith et al. 2011; Garrett et al. 2013). Nocturnal mouse lemurs, in particular, show diversifying selection to expand vomeronasal receptor repertoires (Young et al. 2010; Yoder et al. 2014). Several studies have noted detecting chemical cues in low-light conditions is under strong selection among nocturnal mammals, which have refined and well-developed nasal chemosensory systems and a more discriminating sense of smell (Barton et al. 1995; Wang et al. 2010; Garrett et al. 2013). As some primates evolved diurnality and became more visual, the selective pressure to maintain a well-developed social chemosensory system may have relaxed. This explanation for the loss of vomerolfaction is unlikely to apply for bats, as nearly all bats are nocturnal and most lack vomerolfactory systems.



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*Sensory system exchange did not influence* Trpc2 *loss in bats*

Sensory exchange is another mechanism through which selection can change over time, perhaps shaping the curious patterns of vomerolfaction in bats (Zhao et al. 2011; Jones et al. 2013; Hayden et al. 2014). Maintaining neural sensory tissue is energetically expensive (Niven and Laughlin 2008). If environmental demands select for specialization or adaptation in another sense, natural selection on a less constrained sensory system may relax. The relationship between trichromatic vision in primates and decreased numbers of olfactory receptors is one proposed example of sensory exchange (Gilad et al. 2004), though this example remains contentious (Matsui et al. 2010). Most bats primarily forage using echolocation and many communicate with ultrasonic social calls and have evolved a remarkable diversity of adaptations for acute hearing (Jones et al. 2013), suggesting a sensory exchange. But there is no evidence of decreased reliance on olfaction among echolocating bats, or of decreased importance of echolocation among bats with vomerolfaction. For example, *Tadarida brasiliensis*, an efficient echolocating insectivore, has a disrupted *Trpc2,* but mothers still scent-mark their pups to identify them within the crowded colonies (Gustin and McCracken 1987). Additionally, all miniopterids have well-conserved *Trpc2* genes, but also rely on vocalizations to locate their insectivorous prey (Miller-Butterworth et al. 2007). The evolution of constant-frequency echolocation with Doppler shift compensation is an extreme sensory adaptation that enables focusing on insect prey despite a cluttered background (Fenton et al. 2012). If this adaptation resulted in sensory exchange for vomerolfactory function, the pseudogenization of *Trpc2* should roughly correspond with its evolution. This pattern is not evident: most loss of function mutations occurred among non constant-frequency bats, and *Pteronotus parnellii* —the only New World bat capable of constant-frequency echolocation— still possesses an intact *Trpc2* gene and vomeronasal structures (Bhatnagar and Meisami 1998; Fenton et al. 2012).

Some bats have other extreme sensory adaptations besides echolocation (Jones et al. 2013). Many of these adaptations are found within phyllostomids, one of two families with conserved



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vomerolfaction. For example, the common vampire bat (*Desmodus rotundus*) is the only mammal capable of infrared thermoperception (Gracheva et al. 2011), and UV vision has evolved in the nectarivorous *Glossophaga soricina* (Winter et al. 2003; Müller et al. 2009). Nevertheless, both of these species have well-developed vomeronasal organs and an intact *Trpc2* (Frahm and Bhatnagar 1980; Wible and Bhatnagar 1996; Bhatnagar and Meisami 1998). Similarly, a previous study found no support for an exchange between dichromatic vision and vomeronasal function (Zhao et al. 2011; Jones et al. 2013). Finally, and most surprisingly, phyllostomids with well-developed vomeronasal organs also have larger numbers of distinct olfactory receptors, indicating a lack of exchange between olfaction and vomerolfaction (Hayden et al. 2014). With increased sampling of *Trpc2* among phyllostomid bats with diverse sensory adaptations, we find no support for a simple sensory exchange between vomerolfaction and other senses. Our findings challenge novel environments or sensory exchange as explanations for the loss of vomerolfaction, and open up new interpretations for mechanisms of loss.

*Main olfactory system may compensate for vomeronasal loss*

Compensation between the main olfactory and vomerolfactory system is a hitherto unexplored mechanism for the relaxation of selection on vomerolfaction and its eventual loss. Instead of an increase or expansion in the main olfactory system correlated with vomeronasal loss (a hypothesis rejected by Hayden et al. 2014), olfaction and vomerolfaction could be redundant, allowing selective pressures on vomerolfaction to relax in some species. Recent neurobiological, developmental, and behavioral studies suggest there is more interaction between the two nasal chemosensory systems than previously thought (Suárez et al. 2012; Baum and Cherry 2014; Omura and Mombaerts 2014; Ma 2015). In mice, for example, some pheromone receptors may also be present in the main olfactory epithelium (Omura and Mombaerts 2014; Kanageswaran et al. 2015), possibly explaining why some apparently pheromone-mediated behavior is maintained in species lacking the vomeronasal organ. While *Trpc2* has been pseudogenized in the harbor seal and river otter



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(Yu et al. 2010), both species retain a well-developed main olfactory system and behavioral studies indicate the presence of some olfactory social communication (Van Valkenburgh et al. 2011; Stoffel et al. 2015). Finally, the debate on whether humans are capable of detecting pheromones is still far from resolved, as anatomical advances are ongoing (Smith et al. 2014; Wessels et al. 2014; Vasuki et al. 2016), and behavioral experiments suggest that we can perceive conspecific chemosensory cues (Radulescu and Mujica-Parodi 2013; Lübke and Pause 2015; Gračanin et al. 2016).

We also find similar curious exceptions across bats lacking vomeronasal organs. Despite the gregariousness of many bats—some species reside in caves with millions of individuals (Kunz 1982; Iskali and Zhang 2015), while others in smaller roosts maintain stable relationships among individuals (Kunz et al. 1994)—most lack the vomeronasal organ that mediates social interactions in most mammals. Relying only on the main olfactory system may be sufficient for many animals that spend part of their lives underwater or in the air, as long as some of the chemical detection mechanisms in the vomeronasal and main olfactory systems are redundant. The loss of a sensory system does not preclude processing of the signal in some other way; indeed many "earless" frogs can still detect sound (Lindquist et al. 1998; Boistel et al. 2013). If this hypothesis were correct, the main olfactory system should contain mechanisms to detect pheromone ligands, and express vomeronasal receptors. Functional assays testing for chemosensory redundancy offer a promising avenue for future work.

**Conclusion**

Because of its importance to social communication, vomerolfaction is rarely lost in mammals. Here, we have identified the greatest number of losses of function in this crucial system in any mammalian order, and quantified and modeled the molecular evolutionary history of the key regulator of vomeronasal system function, *Trpc2*. These analyses reveal losses have been independent and varied through time, in contrast with strong and sustained purifying selection in two bat families and their ancestors. Neither shifts to novel environments, nor previously proposed sensory exchanges explain vomerolfactory loss in bats. Instead, we propose the olfactory system may compensate for



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vomeronasal loss, as many bats without *Trpc2* still demonstrate pheromone-mediated behaviors. The molecular evolution of *Trpc2* in bats suggests that, in line with Dollo's law, once vomerolfaction is lost, it does not evolve back.

**Data Accessibility**

Sequences have been deposited on GenBank (KX537508-KX537612). Configuration files for all analyses and details for model implementation are available on Dryad (DOI: http://dx.doi.org/10.5061/dryad.4hk01).

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**Author Contributions**



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LRY conceived the ideas, designed and executed the study, collected and analyzed the data, and wrote the manuscript. RA and CMG assisted in data collection and edited the manuscript. LMD helped design the study, collected samples, and wrote the manuscript. SJR assisted in sample collection. SJR, KES, and ERD were involved in funding acquisition and provided feedback.

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