Evolution of sweet taste perception in hummingbirds by transformation of the ancestral umami receptor

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SENSORY BIOLOGY

Evolution of sweet taste perception in hummingbirds by transformation of the ancestral umami receptor

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Sensory systems define an animal's capacity for perception and can evolve to promote survival in new environmental niches. We have uncovered a noncanonical mechanism for sweet taste perception that evolved in hummingbirds since their divergence from insectivorous swifts, their closest relatives. We observed the widespread absence in birds of an essential subunit (T1R2) of the only known vertebrate sweet receptor, raising questions about how specialized nectar feeders such as hummingbirds sense sugars. Receptor expression studies revealed that the ancestral umami receptor (the T1R1-T1R3 heterodimer) was repurposed in hummingbirds to function as a carbohydrate receptor. Furthermore, the molecular recognition properties of T1R1-T1R3 guided taste behavior in captive and wild hummingbirds. We propose that changing taste receptor function enabled hummingbirds to perceive and use nectar, facilitating the massive radiation of hummingbird species.

Sensory systems display remarkable flexibility across vertebrates, with some animals losing sensory modalities that are no longer key for survival (*1*, *2*) and others evolving new adaptive sensory capabilities (*3*). The repertoires of sensory receptors for odors, pheromones, and tastes reflect species-specific ecology, with receptor families rapidly expanding and contracting (*4*), and in some lineages, new receptor families evolving (*5*). In the olfactory system, functional expansion of the receptor repertoire predominantly involves a pattern of gene duplication and mutation, leading to novel receptors with altered ligand recognition properties (*6*). This pattern of gene duplication and mutation is also observed in vomeronasal receptors and bitter taste receptors, but not in sweet and savory taste receptors (*4*, *7*). Receptors for these palatable tastes are unique among the chemosensory receptor families in that they are highly conserved in number and amino acid identity. New vertebrate sweet receptors, and the evolutionary mechanisms that underlie their acquisition, have not previously been identified.

In vertebrates, sweet and savory ("umami") tastes are sensed by G protein–coupled receptors (GPCRs) termed T1Rs (*8*). Most vertebrates have three T1Rs, with the T1R1-T1R3 heterodimer mediating umami taste and the T1R2-T1R3 heterodimer mediating sweet taste (*8*, *9*). Human T1R2-T1R3 detects carbohydrates and artificial sweeteners (*10*), and knockout mice lacking T1R2 or T1R3 have defective sweet taste perception (*9*, *11*). In genomes analyzed so far, *T1R* expansions are observed only in some fish species (*12*), whereas losses are observed in other vertebrates, often in accordance with diet. Some obligate carnivores, such as cats, lost T1R2 and appetitive behaviors toward carbohydrates (*1*), whereas the giant panda, which feeds predominantly on bamboo, lost T1R1 (*2*). Chickens, turkeys, and zebra finches also do not have T1R2 (*13*), but the relationship between T1R repertoire and avian ecology is unclear. Birds display tremendous heterogeneity in diet, with different lineages primarily consuming fruits, nectars, animals, and seeds. Hummingbirds are specialized nectar feeders, and their ability to perceive and use sugar-rich resources allowed them to colonize a nectarivorous niche, enabling their extensive diversification (*14*). However, how hummingbirds detect sugars remains unknown, so we characterized the repertoires and functions of bird taste receptors to understand the underlying mechanisms of sugar perception.

We identified T1Rs in whole-genome sequences available for 10 birds with different diets and compared them to T1Rs from other vertebrates (Fig. 1A). Also, we cloned T1Rs from the oral tissue of Anna's hummingbirds (*Calypte anna*); the domestic chicken (*Gallus gallus*), which does not prefer sugars (*7*); and the insectivorous chimney swift (*Chaetura pelagica*), because swifts are the closest living relatives of hummingbirds (Fig. 1B and fig. S2). Expression in oral tissue was verified by reverse transcription polymerase chain reaction (fig. S2). Two *T1R* genes—*T1R1* and *T1R3*—were detected in each available bird genome, and candidate signatures of positive selection were identified in the hummingbird lineage (Fig. 1B and table S2), but not the chicken or swift lineages. We failed to detect *T1R2* in bird genomes, despite the presence of flanking loci. Non-avian reptiles retained *T1R2*, including the Chinese alligator (*Alligator sinensis*), a member of the sister group to birds (fig. S1), suggesting that the loss of *T1R2* occurred within Dinosauria. These findings suggest that an alternative T1R2-independent mechanism for sugar detection arose in avian species that display high behavioral affinity for nectar or sweet fruit.

To identify avian sweet receptors, we analyzed responses of bird taste receptors to sugars and amino acids (Fig. 2). Responses of bird T1Rs were measured in heterologous cells by means of calcium-sensitive photoprotein reporters (*15*). Hummingbird T1R1-T1R3 responded to several carbohydrates, including sucrose, fructose, and glucose. Responses were not observed when T1R1 or T1R3 alone was used, suggesting that hummingbird T1R1-T1R3 functions as an obligate heterodimer. Hummingbird T1R1-T1R3 also detected sucralose and various sugar alcohols, including sorbitol and erythritol, but not cyclamate, acesulfame K, and aspartame, which are sweet to humans (*16*). Low-affinity responses were observed to some amino acids, as with the human sweet receptor, which recognizes carbohydrates as well as proteins, dipeptides, and amino acids (*8*). In contrast, cells expressing chicken or swift T1R1-T1R3 failed to detect carbohydrates at any concentration tested and instead recognized alanine and serine. Thus, T1R1-T1R3 heterodimers from swifts, chickens, primates (humans, squirrel monkeys, baboons, and macaques), rodents (mouse and rat), and teleost fish (zebrafish and medaka) detect palatable amino acids (*8*, *17*, *18*). In contrast, in the hummingbird lineage, this receptor complex acquired a new function in the past 42 to 72 million years (*14*, *19*), evolving the capacity for carbohydrate recognition.

Next, we sought to understand the critical changes in hummingbird T1R1-T1R3 that enabled sugar detection. We designed protein chimeras involving portions of hummingbird T1R1-T1R3, which responds to sugars, and chicken T1R1-T1R3, which does not (Fig. 3 and fig. S3). We focused on the venus flytrap domain, an extracellular region of family C GPCRs that mediates ligand binding (*20*). Introducing the venus flytrap domain of chicken T1R3 into hummingbird T1R3 (chimera 1) rendered the heterodimeric receptor sensitive to amino acids rather than sugars. Reintroducing 109 amino acids (residues 158 to 266) of hummingbird T1R3 into the chicken T1R3 venus flytrap domain restored sucrose responses (chimera 2). Further analysis of this 109–amino acid region identified 19 nonconsecutive amino acids (chimera 3; sites: fig. S3), which were collectively sufficient to impart sucrose and sucralose sensitivity (fig. S4). Subsets of these 19 residues did not similarly support sugar binding (fig. S3). Two identified sites (I206 and S237) displayed

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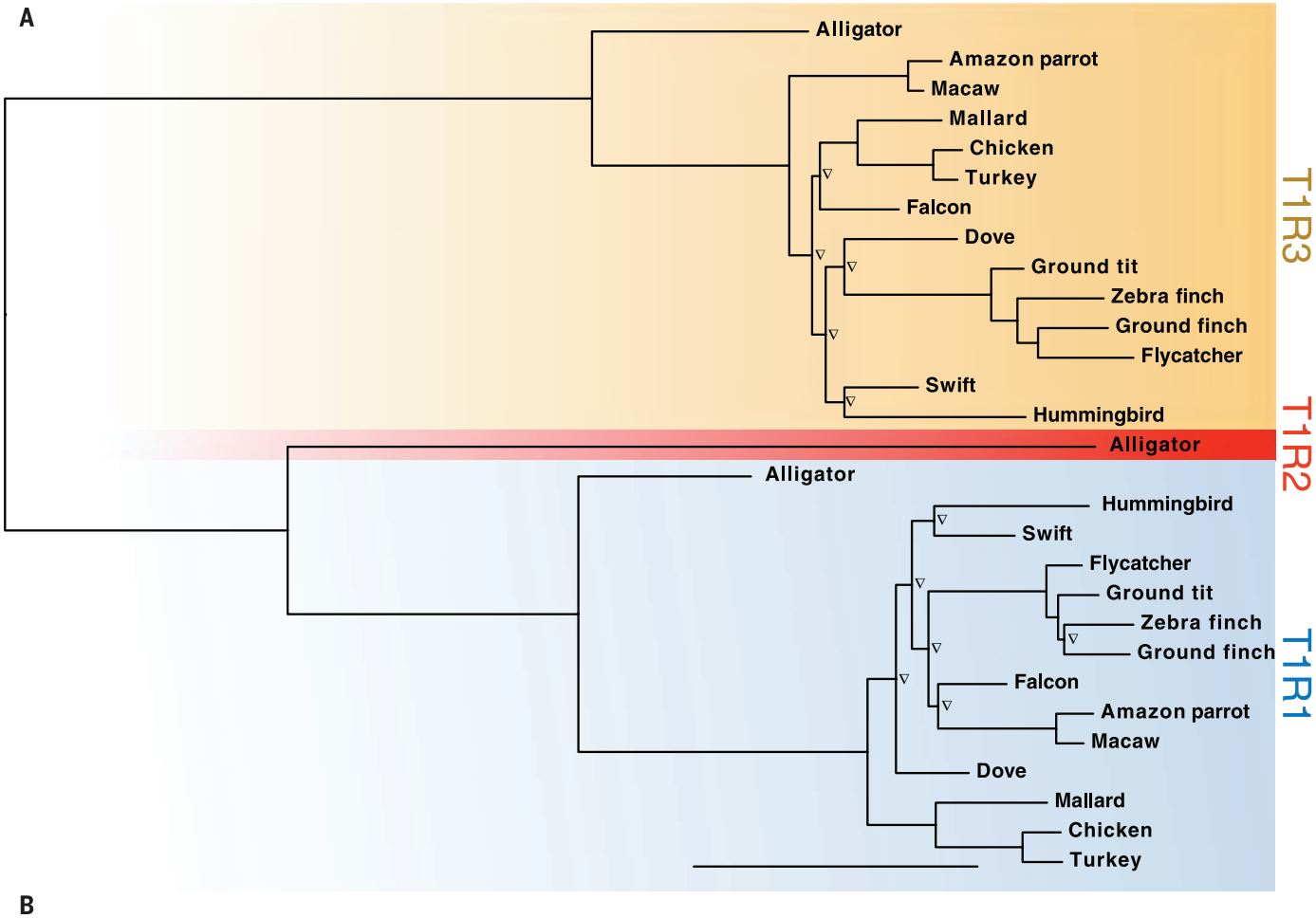
evidence of positive selection (Fig. 1B). Hummingbird T1R1 also contains sites that are under putative positive selection and mutations that contribute to acquired sugar responsiveness, because a mixed receptor pair of chicken T1R1 and hummingbird T1R3 prefers amino acids (fig. S5). Thus, the evolution of carbohydrate detection by hummingbird T1R1-T1R3 involved widespread mutation of both receptor subunits.

We created a homology model of the T1R3 venus flytrap domain based on the x-ray crystal structure of the same region in a related GPCR, metabotropic glutamate receptor 1 (mGluR1) (*20*) (Fig. 3D). This model predicted that the 19 sugar response–conferring substitutions in T1R3 were clustered in three distinct regions of the protein. Three residues (G165, I167, and N211) were in the putative orthosteric ligand-binding site, which in mGluR1 is located at the interface between the two lobes of the venus flytrap domain on the extracellular surface (*20*). G165 and I167 align near S186 and T188 of mGluR1, which form salt bridges to the glutamate ligand (*20*), and all three align near T1R1 residues that are important for ligand responses (*18*, *21*). The remaining residues clustered in two other locations, whose functions in family C GPCRs



B

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                1        10        20        30        40        50        60        70        80        90       100       110       120
Chicken         MIPWVLLCMSFGCAAALKPSCLSAQFRRPGDYIIGGLFPFGMDTINLTARSEPTLIVCERLFVDGLIWALGMKFAIDEINNSTSLLPGVELGYDIYDTCFEPLAALQPSLLFVTQNGTTG
Swift           MVPALLLCWSFGYTAALKPTCLSAQFRRPGDYILGGLFPFGMDTVNLTARSEPSLVVCERLFVEGLIWALGMKFAIDQINNSSSLLPGVQLGYEMHDTCFEPLVALQPSLLLLARGGTRA
Hummingbird     MVPTLLLGLSFGYAAAQETVCLSAQFRSPGDYILGGLFPFGMDIVNLTARTEPTSVKCERLFVDGLIWALGMKFAINEINNSSSLLPGVKLGYDMHDTCFEQVVTLQSSLLFLTQKGTTG
                130       140       150       160       170       180       190       200       210       220       230       240
Chicken         IGIACNYTDYQPRVTAVIGPHKSDLCLLTAKLFSFFLIPQVSYGASSEKLSNKELYPSFYRTVPSDKNLVEAVVLLLDEFGWNWIATIGSDDEYGRGAQELFLSTIGNSSICIAYEGLIP
Swift           VAPLCNHSSYQPRVTAVIGPHKSGLCLLTAKLFSSFLIPQVSYAASSEMLSNTNLYPSFYRTVPTDKNLVEAVVLLLKEFGWNWIATIGSDDEYGRGAQGLFLSIAGNHSICIAFEGLIP
Hummingbird     IEVSCNYTNYQPRVTAVIGPYKSDLCLLTAKLFSFFLLPQISYGGSIEKLSNTDSYPSFYRTVPGDNNLVEAVAQLLNKFGWNWIITIGSNDEYGRGAQGHFVSIAENRNICIAFEGLIP
                250       260       270       280       290       300       310       320       330       340       350       360
Chicken         SDLTDPRAEKQLEETIQYINKTNVNIIVLFAFRQPAQALLEQSIKMRLSKKVWIGTEAWLLSDIAASIPNIQNIGTVLGFIMKASTVPGFQKYVANLLSSVQQDEFCQKSREFYRHVSSD
Swift           TDLADPKAKKQLEDTIKSINRTKVNVIVLFAFSHPAQALLEHSIRMGLSKKVWIGTEAWILSDIIASIPNIQSIGTVLGFIIKTGTVPGFQQYVANLFTSIQQDKFCQESREFNQLMNSE
Hummingbird     TDLADPKTTNQLENIIKAINKTDANVIVLFAFSQTALALLEHSIRMGLSKRVWIGTETWTLSHKAASISNIQSIGTVLGFVMKAGTVPGFHKYVTDLFSSAQHDEFCQQSRKSNHLMSSD
                370       380       390       400       410       420       430       440       450       460       470       480
Chicken         TLGTQCQQCDHISLNDISSTLSHSQIQPVYIAVYSVAYALHRALGCTHQGCPRASIRSWQLLHFMNTVPFTVNGQSFRFDESHGTNSGYNLIFWHWENGSLTHLPVGDYQESLYLNKSLI
Swift           VLDTPCEQCDHISLHQVLSTLSHSQVQPVHIAVHSVATALHRALGCTHQACPKAPVRPWQLLHFMNTLPFQVNGQSFRFDQSHGTNTGYQLMFWSWKNSTLTYLPVGDYEESLYINKSQI
Hummingbird     VLSTPCEQCDYATLPQILPTLNHLKIIPVYLAVYSVANALHRALGCTHQACPKAPLKSWQLLHFMNTFPFTVNGQSFRFDKFHGTNPGYQLIFWLWRNGTLEYLPVGEYKESLAINVSQI
                490       500       510       520       530       540       550       560       570       580       590       600
Chicken         QFHTTDQKEPTSECFRCEPGQIRQIKGFHICCYDCTDCPENTFCSSKDSSTCTPCLEHQWSPARSTQCYDRSERYLRWNEPLTAGLLISMSIIISLICLTAVLFVKNLNTPLVQAAGGN
Swift           QFHTADQKEPTSECFRDCSPGQFRQIKGFHLCCYDCTDCPENTFWSSKDSSSCSPCPQQHWAPARSTRCHLRSDRFLFWSEPLSIALLTLMSITLSLTCLTALLFFKSLETPLVQASGGK
Hummingbird     QFHTPNQERPSSQCFAQCLPGQIRQIKGLHMCCYDCRDCPENTFSSAKDQSRCTSCQKQQWAPTSSIQCYDRSERYLFWNEPLTIALLLGMSLTLSLTCATALIFLKNLETPLVQASGGK
                610       620       630       640       650       660       670       680       690       700       710       720
Chicken         LNLFALFALTLMCLSSCLFIGKPTNNLCMMQQIVCALCLNACFSTFFIKSLEIVLLTEFPRCARTALRWVTPSRSWLLVALCLLTECLFCFCYLHLGPDYVLPDYSSLPTEVLLMCSTAS
Swift           LSLFALFALVLLCLSCCLYIGKPSHHLCMTQQMVHALCLNGCFSTFFIKSLEITLVTEFPRCAPTFLPWLTQRRAWLLVALCLLTQALLCLCYLHVGPDYLLSDYKSLPTEVLLVCDTRS
Hummingbird     LNLFGLFMLTLQSLSCCLYVGKPSDNLCTIQQIVYALCLNGSFSTFFIKSLEIILVTEFPRCAPTFLPWLTQRRAWLLVALCLLIQSFLSFCYVQLGPDYLQADYESLRSEVLLVCDTKS
                730       740       750       760       770       780       790       800       810       820       830       836
Chicken         WPAFALMHGYNGCLAFVCFLCTFMVQSSGKKYNMARGITFTILIYFIIWIFFITVFATLRTVLMSVIQISTILMVSLGIVGTYYIPKCYILLLKPDLNREDYFQYSTKEEPEGD
Swift           WFAFTLLHGYNSCLAFVSFLGTFMVQTSSRKYNIARGITFAILIYFIIWIFFIAIFVTLKTVLRSVTQIGTILTTTLGIIGTYYIPKCYILLLKPDLNTVDYFQNSIKEEPGEDSQ
Hummingbird     WFAFTLLQGYNCCLAFVCFLCTFMVQTSGKQYNIARGITFAILTYFIIWIFFVAIFTTLKTVLRPATQMGTILATSLGIIGTYYIPKCYLILLKPELNKVDYFQHSIKEEPDEDSQ
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Fig. 1. Analysis of T1R sequences in birds. (**A**) A maximum-likelihood tree was constructed using *T1R* sequences from 13 birds and the Chinese alligator (∇ = nodal bootstrap <80%; scale bar, 0.4 substitutions per site). (**B**) Amino acid sequences of T1R3 cloned from birds. Gray, transmembrane domains; red, putatively selected sites (table S3).

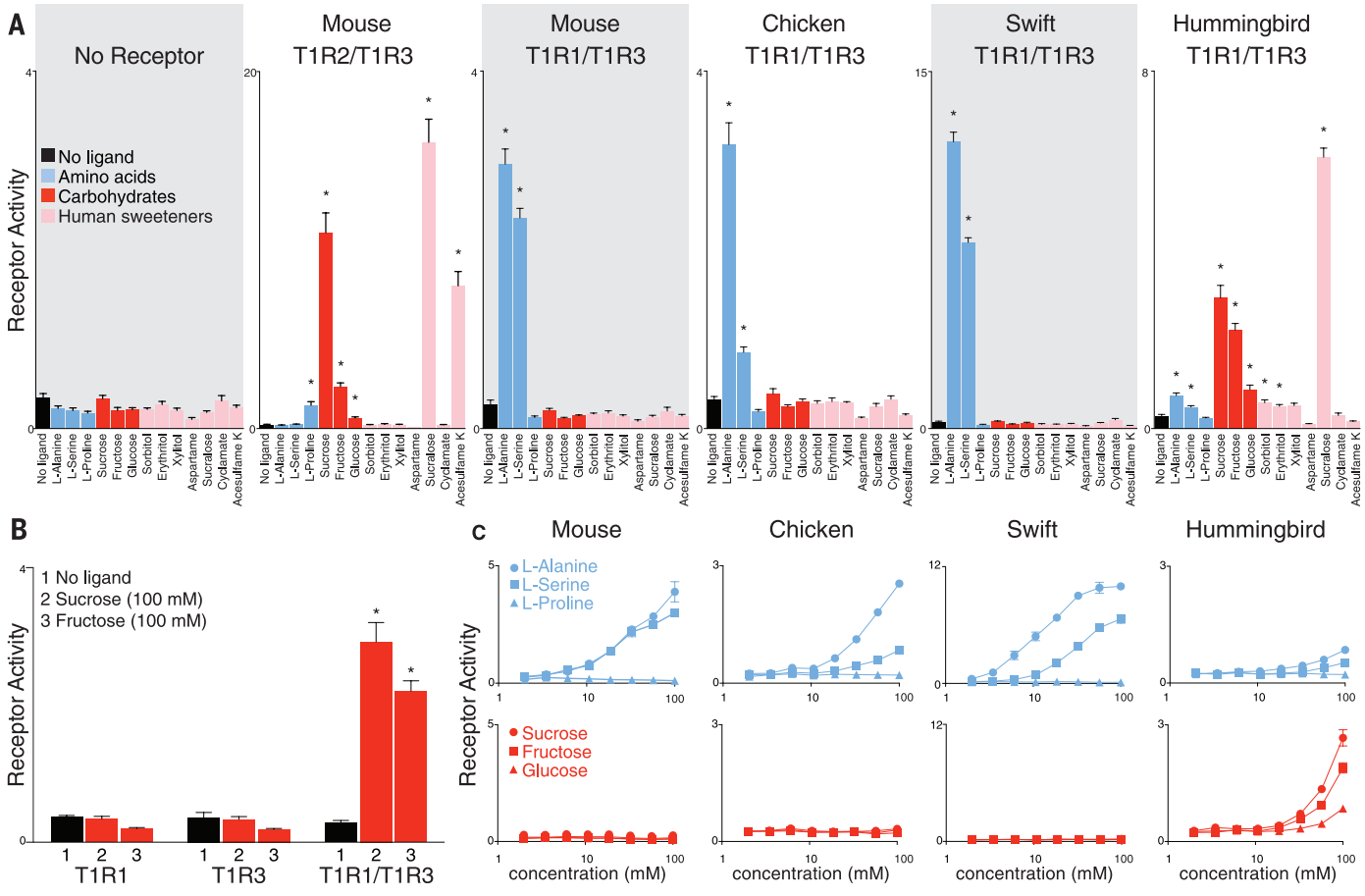

Fig. 2. Evolution of a sugar receptor in hummingbirds. (**A**) Functional expression of avian and rodent taste receptors to stimuli [100 mM, except aspartame (15 mM); n = 6 independent experiments, mean ± SE, *$P \leq 0.05$]. (**B**) Sugar responses of hummingbird T1Rs alone or in combination (n = 6 independent experiments, mean ± SE, *$P \leq 0.05$). (**C**) Dose-dependent responses of T1R1-T1R3 from species indicated to amino acids (blue) and sugars (red).

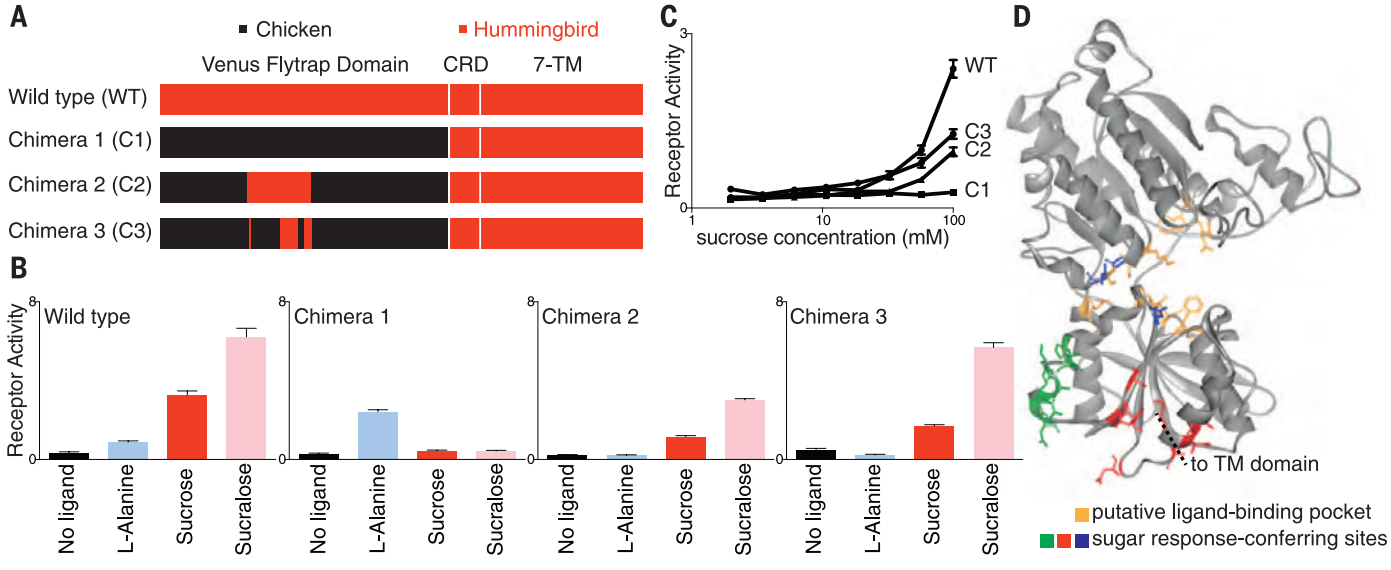

Fig. 3. Molecular basis for the acquisition of sugar binding in hummingbird T1R1-T1R3. (**A**) T1R3 chimeras containing chicken (black) and hummingbird (red) amino acids were designed (CRD, cysteine-rich domain; TM, transmembrane domains). (**B**) Responses of T1R3 chimeras and hummingbird T1R1 to L-alanine, sucralose, and sucrose (100 mM). (**C**) Dose-dependent responses of T1R3 chimeras and hummingbird T1R1 to sucrose. (**D**) A homology model of the venus flytrap domain of T1R3 shows the putative ligand binding site (yellow), predicted by alignment with ligand-contacting sites of rat mGluR1 (*20*), and mutations that confer sugar binding, which cluster in three distinct locations (red, green, and blue).

are unknown but may be important for folding topology, interdomain or intersubunit interactions, or G protein activation. The dramatic redecoration of the T1R1-T1R3 protein surface that occurred in hummingbirds to allow for sugar binding makes sense, given that carbohydrates and amino acids adopt completely different structures.

We next asked whether T1R1-T1R3 function would dictate hummingbird taste behavior. We reasoned that nonnutritive agonists of T1R1-T1R3 without caloric value would be palatable to hummingbirds, like artificial sweeteners are to humans. Hummingbirds prefer sugars (*22*), but behavioral responses to many other human sweeteners are unknown. We developed a brief-access, two-choice gustatory preference paradigm in captive ruby-throated hummingbirds (*Archilochus colubris*) to measure taste responses to T1R1-T1R3 ligands (Fig. 4A). As expected, hummingbirds displayed strong behavioral affinity for sucrose over water, as measured by an increase in mean drinking bout length, number of long bouts (>1 s of uninterrupted drinking), and overall time spent drinking. High-speed video recordings (movie S1) indicated extremely rapid choice decisions; water trials terminated within three or four tongue licks (~250 ms), suggesting that sugar preference involves rapid processing of taste information rather than post-ingestive effects. Ruby-throated hummingbirds equally consumed solutions of sucrose and erythritol, a nonnutritive agonist of hummingbird T1R1-T1R3, but displayed a strong preference for sucrose over aspartame, a sweetener to humans that failed to activate hummingbird T1R1-T1R3.

We also developed a behavioral assay involving Anna's hummingbirds because we cloned T1R1 and T1R3 from this species (Fig. 4B and movie

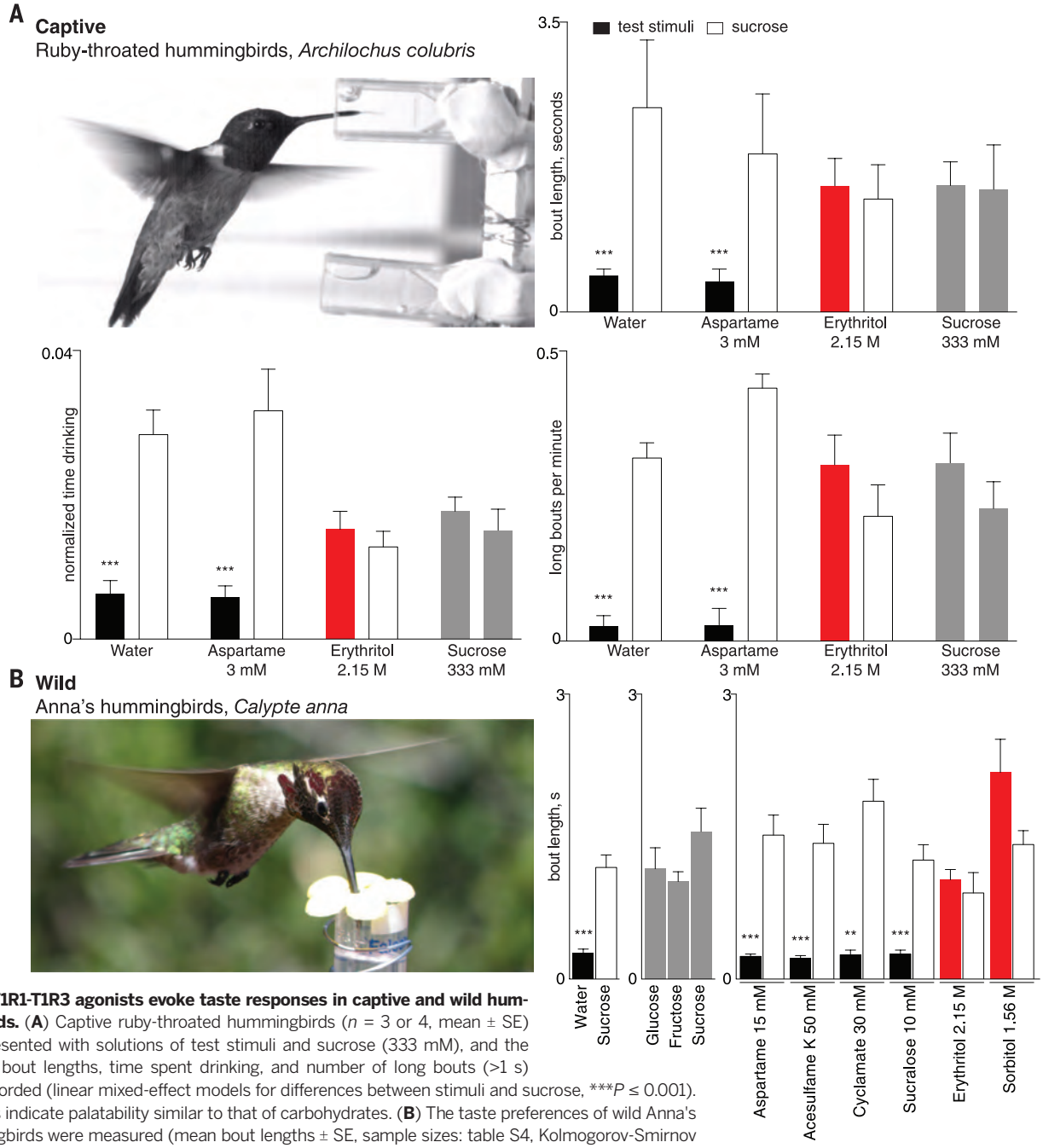

Fig. 4. T1R1-T1R3 agonists evoke taste responses in captive and wild hummingbirds. (**A**) Captive ruby-throated hummingbirds (n = 3 or 4, mean ± SE) were presented with solutions of test stimuli and sucrose (333 mM), and the drinking bout lengths, time spent drinking, and number of long bouts (>1 s) were recorded (linear mixed-effect models for differences between stimuli and sucrose, $^{***}P \leq 0.001$). Red bars indicate palatability similar to that of carbohydrates. (**B**) The taste preferences of wild Anna's hummingbirds were measured (mean bout lengths ± SE, sample sizes: table S4, Kolmogorov-Smirnov tests for differences between stimuli and sucrose: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$). Concentrations: white, 500 mM; gray, 1 M; black, indicated. Red bars indicate equal preference. [Photo credits: (A) M.W.B. and F. Peaudecerf, (B) M.W.B.]

S2). Experiments were performed in the Santa Monica Mountains at a field site frequented by wild hummingbirds. We recorded the behavior of birds presented for 15 min with test stimuli, and in control experiments, hummingbirds displayed strong preference for sucrose over water and high behavioral affinity for several sugars abundant in nectar, including sucrose, glucose, and fructose. Next, we presented solutions of sucrose and test stimuli, and measured the mean drinking bout length. Anna's hummingbirds displayed a strong behavioral attraction to the T1R1-T1R3 agonists erythritol and sorbitol, with responses similar to those to sucrose. In contrast, Anna's hummingbirds displayed a strong preference for sucrose over other structurally diverse human sweeteners that failed to activate hummingbird T1R1-T1R3, including aspartame, cyclamate, and acesulfame K. Furthermore, these synthetic human sweeteners were aversive at high concentrations, because birds rejected mixed solutions containing these chemicals and sucrose (fig. S6 and movie S2) and often displayed a characteristic behavioral pattern involving beak withdrawal, head shaking, and/or spitting that was previously observed in response to the ingestion of bitter plant metabolites (*23*). This reaction was also observed toward sucralose solutions, and mixtures of sucrose and sucralose were not consumed (fig. S6), indicating that sucralose is also actively rejected. Other species of hummingbirds (black-chinned and Allen's hummingbirds) visited and displayed similar taste preferences (fig. S6). Together, these behavioral experiments show that several agonists of hummingbird T1R1-T1R3, including simple sugars and sugar alcohols, evoke fast, appetitive gustatory responses in hummingbirds. Other synthetic human sweeteners that do not activate hummingbird T1R1-T1R3 are not similarly attractive and are often actively rejected. We conclude that the molecular recognition properties of hummingbird T1R1-T1R3, together with those of other gustatory receptors, instruct taste behavior in both captive and wild hummingbirds.

We studied the repertoire and function of taste receptors to provide a molecular basis for variations in animal ecology and the evolutionary events that cause them. We identified a transformation of taste receptor function that occurred in hummingbirds after their divergence from an insectivorous ancestor. We propose this to be a key evolutionary adaptation that contributed to the acquisition of nectar-feeding behavior and enabled the extensive radiation of hummingbird species. The molecular basis for this change in taste behavior is an altered ligand-binding preference of T1R1-T1R3 from amino acids to carbohydrates, a complex feat that involved dramatic structural changes in the receptor surface. It has been proposed that the ancestral T1R heterodimer, as well as the ancestral family C GPCR, were amino acid receptors (*17*, *24*). The mammalian sweet receptor probably derived from a similar transformation that occurred earlier in vertebrates. Birds descended from carnivorous theropod dinosaurs (*25*), and like mammalian carnivores, it appears that an ancestor of birds lost T1R2, perhaps another example of the close relationship between diet and taste receptor repertoire. Based on evidence presented here, hummingbirds recently evolved a new sugar receptor and consequently regained sweet taste perception.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/345/6199/929/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S6
Tables S1 to S4
References (*26*–*44*)
Movies S1 and S2

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PLANT DEVELOPMENT

Arabidopsis NAC45/86 direct sieve element morphogenesis culminating in enucleation

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Photoassimilates such as sugars are transported through phloem sieve element cells in plants. Adapted for effective transport, sieve elements develop as enucleated living cells. We used electron microscope imaging and three-dimensional reconstruction to follow sieve element morphogenesis in *Arabidopsis*. We show that sieve element differentiation involves enucleation, in which the nuclear contents are released and degraded in the cytoplasm at the same time as other organelles are rearranged and the cytosol is degraded. These cellular reorganizations are orchestrated by the genetically redundant NAC domain–containing transcription factors, NAC45 and NAC86 (NAC45/86). Among the NAC45/86 targets, we identified a family of genes required for enucleation that encode proteins with nuclease domains. Thus, sieve elements differentiate through a specialized autolysis mechanism.

Long-distant transport sustains life in multicellular organisms. In plants, phloem sieve element cells form a transport network specialized for long-distance allocation of photoassimilates and signaling molecules (*1*). Unlike in the animal circulatory system, contents are transported through cells rather than between cells. Differentiation of sieve elements elaborates specialized structures (such as sieve plates with pores) and eliminates others (vacuoles,

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Supplementary Materials for

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Movies S1 and S2

Materials and Methods

Bird specimens

All specimens were obtained after securing appropriate federal and state permits. Domestic chickens (breed: single comb white leghorns) were obtained from the breeding flock of the Department of Animal Science, UC Davis. Wild Anna's hummingbirds (*Calypte anna*) were donated after euthanasia by the Wildlife Care Association in McClellan, CA. A chimney swift (*Chaetura pelagica*) was collected in the field in Cambridge, MA. Specimen collections and behavioral experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Harvard University. Bird tissues are accessioned (Museum of Comparative Zoology, Harvard University; Anna's hummingbird: MCZ 363253, chimney swift: MCZ 363256; chicken: MCZ 363257).

Cloning bird T1R sequences

First, we determined full-length *T1R* coding sequences from hummingbirds, chickens, and swifts. Whole genome sequences were not available for hummingbirds and swifts, and we observed a large gap in the chicken *T1R1* gene, so full length sequences were obtained using a combination of degenerate PCR, RACE (Rapid Amplification of cDNA ends) (*26*), and genome walking. First, we identified internal *T1R* sequences using whole genome data or degenerate PCR involving primers derived from *T1R* genes in other species. For RACE, RNA was extracted from rapidly frozen bird oral tissue (palate and tongue) using RNeasy Fibrous Tissue Kit (Qiagen), and used as a template for cDNA synthesis. 5' and 3' sequences were obtained using the SMARTer RACE cDNA Amplification Kit (Clontech). For genome walking, DNA was extracted from breast muscle using DNeasy Blood & Tissue Kit (Qiagen) and sequences were obtained using the Genome Walker Universal Kit (Clontech). Full-length *T1R* cDNAs were then obtained by PCR from oral tissue cDNA, and cloned into the pEAK10 vector for expression in mammalian cells (*18*). PCR reactions involved Advantage GC 2 Polymerase (Clontech) and annealing temperatures optimized for GC rich sequences. Chimeric T1Rs were prepared by PCR as previously described (*18*).

T1R phylogenetic analysis

We assembled a dataset of 68 predicted *T1R* coding sequences (Table S1) by analysis of online databases or by experimental protocols described above for hummingbirds, swifts, and chickens. Online sequences were obtained either from Ensembl (*27*), GenBank (*28*), or (for birds and alligators) by BLAST analysis (*29*) of available genomic resources. BLAST analysis involved queries using each exon of hummingbird *T1R1* and *T1R3*, followed by manual compilation of BLAST hits to obtain predicted coding sequences. Partial sequences were used when gaps in the available genomic data precluded full sequence determination. In a few genes (zebra finch *T1R1*, ground finch *T1R1*, falcon *T1R1*, and mallard *T1R3*) we observed unusual insertions or stop codons which could reflect pseudogenization or database errors. We did not detect *T1R2* in bird genomes by BLAST analysis, with queries involving human *T1R2* instead retrieving other family C GPCRs, including *T1R1*, *T1R3*, *CaSR*, *mGluR2*, and *GPR6*. By contrast, alligator *T1R2* was readily identified by this approach. Two loci that flank

mouse *T1R2* (*Pax7* and *Aldh4a1*) are syntenic in chicken (*7*), and we observed them to be retained in all 10 bird genomes indicating that this region of the genome was represented in available sequencing data.

We aligned all 68 *T1R* sequences, or subsets of them, using the TranslatorX (*30*) server and the MAFFT alignment program (*31*). Maximum likelihood phylogenetic trees were constructed using PHYML 3.0 (*32*) and JTT+I+G as the most appropriate model of evolution (determined *a priori* using ModelGenerator v0.85 (*33*)). Nodal supports were assessed with 1000 bootstrap replicates. For analyses of positive selection, we used the program CODEML in the PAML package, version 4.4b (*34*). For PAML analysis, we used a tree for *T1R1* and *T1R3* that corresponded to the known species tree. Branch-site models (*35*) and Bayes Empirical Bayes (BEB) analysis (*36*) were used to identify positive selection in specific lineages and at specific sites, as recently described (*37*). Models were evaluated using likelihood ratio tests (LRTs) and χ^2 tests of significance (Table S2). In model 1 (neutral model), codons are allowed to evolve either neutrally (ω or the ratio of non-synonymous substitutions per nonsynonymous site to synonymous substitutions per synonymous site = 1) or under purifying ($0 < \omega < 1$) selection. In model A, sites in specified lineages are allowed to evolve under positive selection ($\omega > 1$). Proportions of sites (p_0, p_1, p_{2a}, and p_{2b}) corresponding to each ω category, and the associated ω values in the specific lineage, are listed in Table S2. Lineage-specific variation in selective pressure was identified in hummingbird T1R1 and T1R3 and evidence for positive selection was supported by comparing model A with the neutral model (model 1), and 6 codons in each gene were identified as positively selected with posterior probabilities > 0.5 (Fig. 1B, Fig. S2, Table S3). The additional required LRT with the null model for model A (*38*) was not significant and we could not rule out relaxed selection rather than positive selection in the hummingbird lineages with purifying selection in others with this test.

T1R functional assay

T1R responses were measured in heterologous cells using a luminescence assay involving a calcium-dependent photoprotein, as previously described (*18*). HEK293T cells were transiently co-transfected with plasmids encoding taste receptors, mouse $G\alpha_{15}$, and mt-apoclytin-II (*18*), exposed to test stimuli, and assayed for luminescence. Ligand-evoked responses were compared with control responses (no ligand), and statistically significant increases ($^*p \leq 0.05$) were determined using Welch's *t*-tests and followed by the Holm adjustment for multiple comparisons ($\alpha=0.05$).

Structural modeling

The homology model of the venus flytrap domain was constructed using the crystal structure of mGluR1 (open form) as a template (PDB ID: 1EWT) as described previously (*18, 20, 39*). Alignment and homology modeling were performed with MOE (Molecular Operating Environment, Chemical Computing Group, Inc.) and visualized using Discovery Studio Visualizer (Accelrys) software.

Behavior of captive birds

Wild-caught ruby-throated hummingbirds (*Archilochus colubris)* were maintained in temporary captivity at the Concord Field Station (Department of Organismic and

Evolutionary Biology, Harvard; Bedford, Mass) and were used for high-speed video recordings and brief-access behavioral trials.

During behavioral trials, birds (n=3-4) were presented simultaneously with two filled cuvettes (2 ml), one containing test stimuli and a second containing sucrose (333 mM). Test stimuli included water, aspartame (3 mM), erythritol (2.15 M), and sucrose (333 mM); these concentrations of aspartame and erythritol are as sweet to humans as 500 mM sucrose (*16*). Behavioral responses were filmed (30 frames per second) for ~5 minutes after the first drink was taken. In between trials, birds were given sucrose in both cuvettes to prevent a side bias from developing, and to ensure that an interest in feeding persisted. Birds were tested multiple times (2-8), with stimuli placed on alternate sides; levels of sucrose consumption were similar in subsequent tests controlling for potential changes in hunger status. Drinking bouts were scored as the time between drinking onset, as defined when the bill tip entered the feeder, and the initiation of withdrawal behavior. For each bird, three parameters of drinking behavior were calculated: mean drinking bout length, percentage of time spent drinking, and number of long bouts (> 1 second) per minute. For Fig. 4A, each parameter is averaged across birds, with a value for each bird determined on a per trial basis. For statistical analysis of Fig. 4A, linear mixed-effects models were used to compare stimulus/sucrose responses per trial (3-4 birds, 23-93 observations, ***$p \leq 0.001$, p-values corrected for multiple testing by the Holm adjustment ($\alpha=0.05$)).

High-speed video recordings were used to measure discrimination speed. Birds (n=5) were presented simultaneously with water and sucrose solution (500 mM), and their behavior was filmed with a Photron 1280 camera at 500 frames per second (Movie S1). The number of licks required for rejection of water was recorded as a mean per bird over 2-3 trials. Water rejection occurred within a mean of 250 milliseconds, and involved 3-4 tongue licks with occasional pauses.

Behavior of wild birds

The taste preferences of wild Anna's hummingbirds were assessed in the field, at a site in the Santa Monica Mountains in Topanga, California, USA. Birds were given brief access to a circular feeder array containing 6 stimuli (3 in duplicate). Stimuli (4 ml) were presented in disposable tubes fitted with wire-secured flower caps from commercial hummingbird feeders. Stimulus presentation was designed so that birds fed while hovering (Movie S2). Feeders contained either (A) water, sucrose (250 mM) and sucrose (500 mM), (B) glucose (1 M), fructose (1 M) and sucrose (1 M), or (C) test stimuli, sucrose (500 mM), and a mixture of test stimuli and sucrose (500 mM). Test stimuli included aspartame (15 mM), acesulfame K (50 mM), cyclamate (30 mM), sucralose (10 mM), erythritol (2.15 M), sorbitol (1.56 M), or other concentrations listed in Fig. S6. Sucrose concentrations in A were used to establish appropriate test conditions. Concentrations in B were used since simultaneous presentation of glucose, sucrose, and fructose at 500 mM resulted in a preference for sucrose, presumably due to the higher affinity of T1R1-T1R3 for this carbohydrate. To control for position effects, each stimulus was duplicated and the array was rotated half-way through the trial. Between each trial, feeders were filled with sucrose solution. Fifteen minute behavioral trials were filmed at 60 frames per second. Three species of hummingbirds- Anna's hummingbirds (*Calypte anna*), black-chinned hummingbirds (*Archilochus alexandri*), and occasionally

Allen's hummingbirds (*Selasphorus sasin*)- visited the feeders, typically with up to 3 male Anna's hummingbirds feeding at once and often > 10 birds near the feeder at a time. Individuals were not color banded, so each visit was treated as a sample. Drinking bout lengths of male Anna's hummingbirds were recorded for test stimuli (Fig. 4B), sucrose (Fig. 4B), and the mixture (Fig. S6) and were scored as above. Bout lengths for all birds (both sexes and all species) were also measured for either the first 7 minutes or the first 200 feeder visits in each trial (Fig. S6). Birds typically sampled briefly from multiple feeders with rarer long bouts (one second or longer) from specific feeders, so statistical analyses were performed using Kolmogorov-Smirnov tests (depicted) and Mann-Whitney *U* tests (similar results). Experiments involving sucralose (1 mM) were tested in a subsequent field season. One bird with a deformed bill and aberrant drinking behavior was removed from analysis of Fig. S6, sucralose, 1 mM.

Supplementary Text

Full acknowledgments

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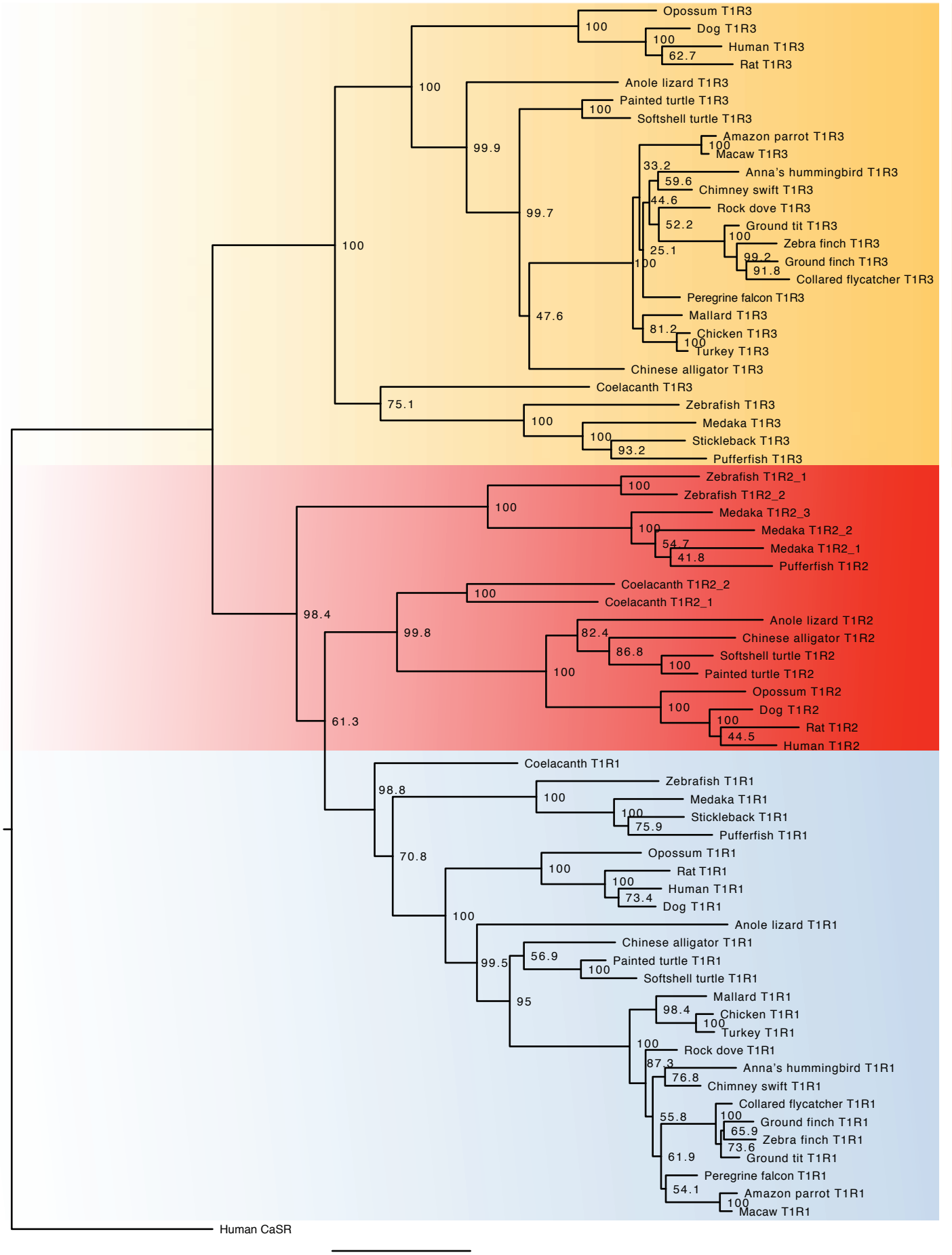
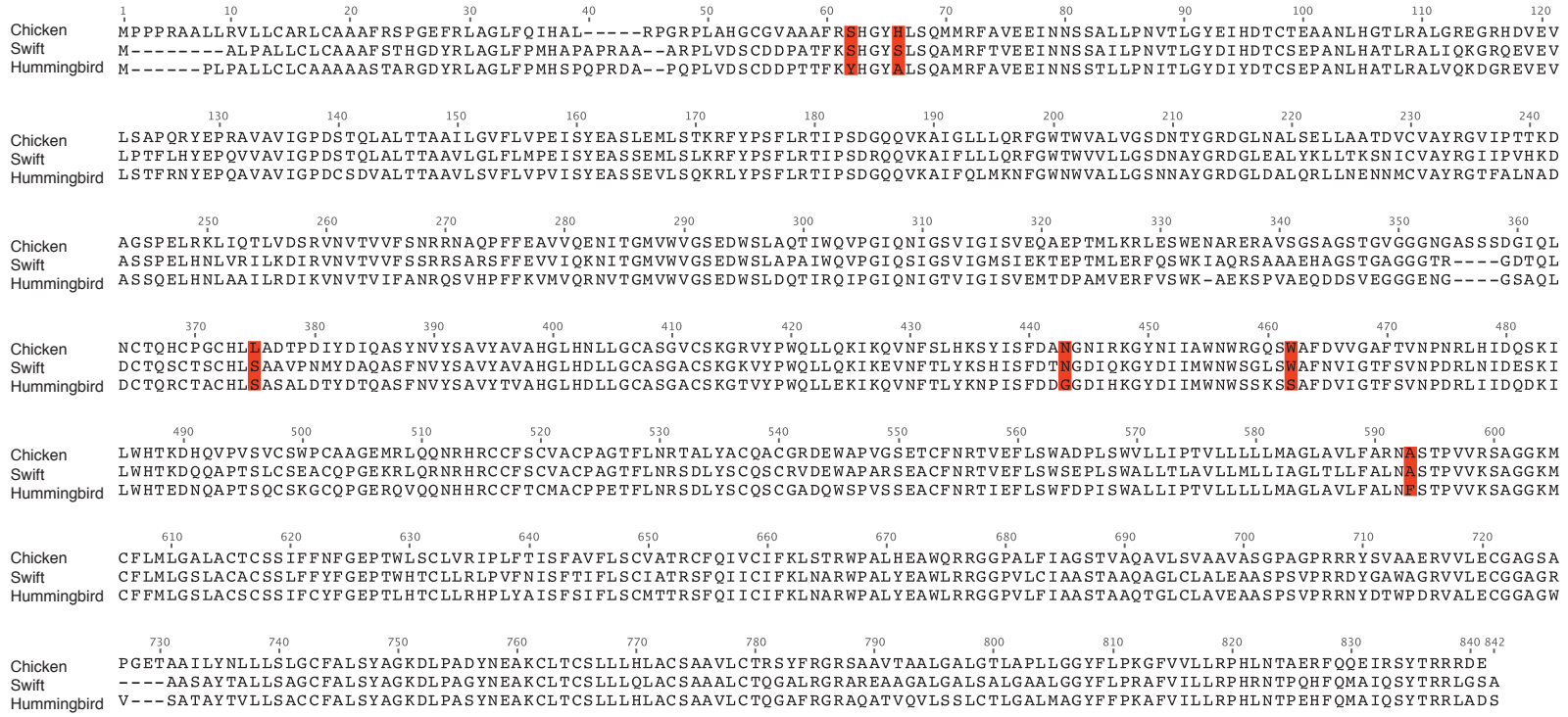

T1R3
T1R2
T1R1
Opossum T1R3
Dog T1R3
Human T1R3
Rat T1R3
Anole lizard T1R3
Painted turtle T1R3
Softshell turtle T1R3
Amazon parrot T1R3
Macaw T1R3
Anna's hummingbird T1R3
Chimney swift T1R3
Rock dove T1R3
Ground tit T1R3
Zebra finch T1R3
Ground finch T1R3
Collared flycatcher T1R3
Peregrine falcon T1R3
Mallard T1R3
Chicken T1R3
Turkey T1R3
Chinese alligator T1R3
Coelacanth T1R3
Zebrafish T1R3
Medaka T1R3
Stickleback T1R3
Pufferfish T1R3
Zebrafish T1R2_1
Zebrafish T1R2_2
Medaka T1R2_3
Medaka T1R2_2
Medaka T1R2_1
Pufferfish T1R2
Coelacanth T1R2_2
Coelacanth T1R2_1
Anole lizard T1R2
Chinese alligator T1R2
Softshell turtle T1R2
Painted turtle T1R2
Opossum T1R2
Dog T1R2
Rat T1R2
Human T1R2
Coelacanth T1R1
Zebrafish T1R1
Medaka T1R1
Stickleback T1R1
Pufferfish T1R1
Opossum T1R1
Rat T1R1
Human T1R1
Dog T1R1
Anole lizard T1R1
Chinese alligator T1R1
Painted turtle T1R1
Softshell turtle T1R1
Mallard T1R1
Chicken T1R1
Turkey T1R1
Rock dove T1R1
Anna's hummingbird T1R1
Chimney swift T1R1
Collared flycatcher T1R1
Ground finch T1R1
Zebra finch T1R1
Ground tit T1R1
Peregrine falcon T1R1
Amazon parrot T1R1
Macaw T1R1
Human CaSR

Figure S1

Fig. S1

Phylogenetic tree of T1Rs from birds and other vertebrates. Maximum likelihood tree of 68 T1Rs from 26 vertebrates was constructed and rooted using human Calcium Sensing Receptor (CaSR), scale bar = 0.4 substitutions per site.

A


T1R1
Chicken
Swift
Hummingbird

B

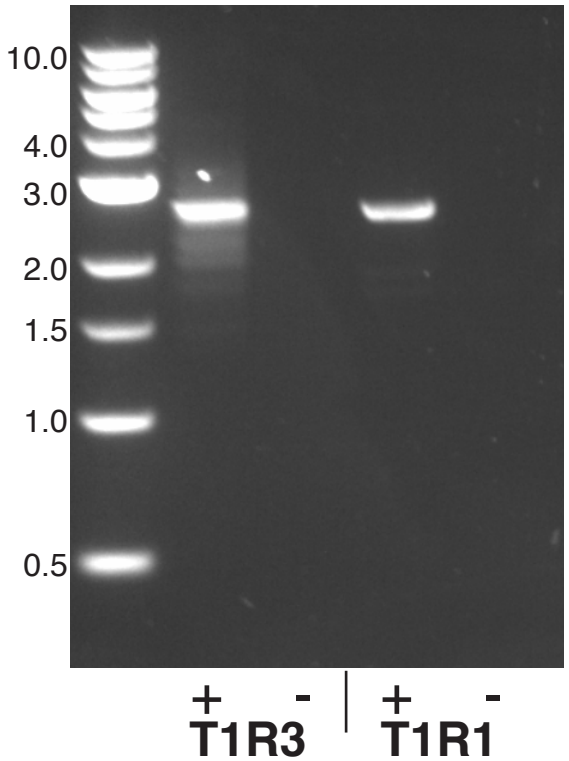

10.0
4.0
3.0
2.0
1.5
1.0
0.5
+
-
+
-
T1R3
T1R1

Figure S2

Fig. S2

Sequences of *T1R1* cloned from hummingbird, chicken, and swift and T1R expression data. (A) Hummingbird, chicken, and swift T1R1 genes were aligned, with red highlighting indicating candidate sites of positive selection (posterior probability > 0.5, Table S3). **(B)** Amplification of full-length T1R1 and T1R3 coding sequence from hummingbird oral tissue cDNA. cDNA reactions were prepared with (+) or without (-) reverse transcriptase.

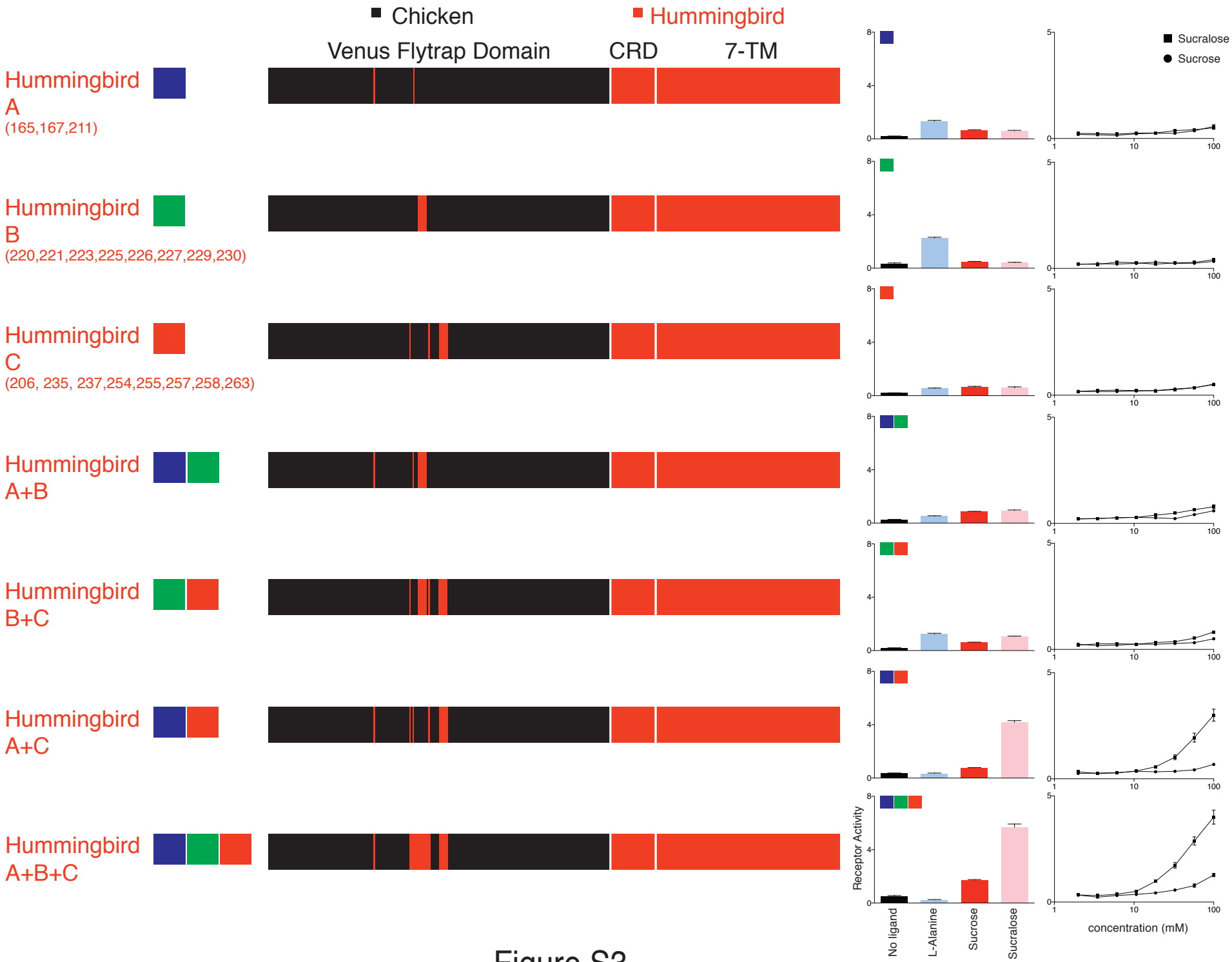

Chicken
Hummingbird
Venus Flytrap Domain
CRD
7-TM
Hummingbird A
(165,167,211)
Hummingbird B
(220,221,223,225,226,227,229,230)
Hummingbird C
(206, 235, 237,254,255,257,258,263)
Hummingbird A+B
Hummingbird B+C
Hummingbird A+C
Hummingbird A+B+C
Sucralose
Sucrose
Receptor Activity
No ligand
L-Alanine
Sucrose
Sucralose
concentration (mM)

Figure S3

Fig. S3

Responses of T1R3 chimeras to tastants. T1R3 chimeras were designed to contain hummingbird (red) and chicken (black) amino acids. The 19 hummingbird amino acids in the venus flytrap domain which conferred sugar responsiveness were confined to three different regions of the protein (Fig. 3), as indicated by the blue square (amino acids 165, 167, 211, near putative ligand contact site), green square (amino acids 220-221, 223, 225-227, 229-230), and red square (amino acids 206, 235, 237, 254-255, 257-258, 263). Cells expressing T1R3 chimeras containing hummingbird-derived amino acids in 1, 2, or all 3 of these regions, and hummingbird T1R1 were assayed for responses to L-alanine, sucrose, and sucralose (each 100 mM, n=6, mean ± SE). Dose-dependent responses were obtained to sucrose and sucralose for each chimeric T1R3 paired with hummingbird T1R1. T1R3 chimeras containing hummingbird-derived amino acids in only one or two of these regions did not similarly respond to sucrose, indicating that all 3 regions contributed to the acquisition of sugar binding.

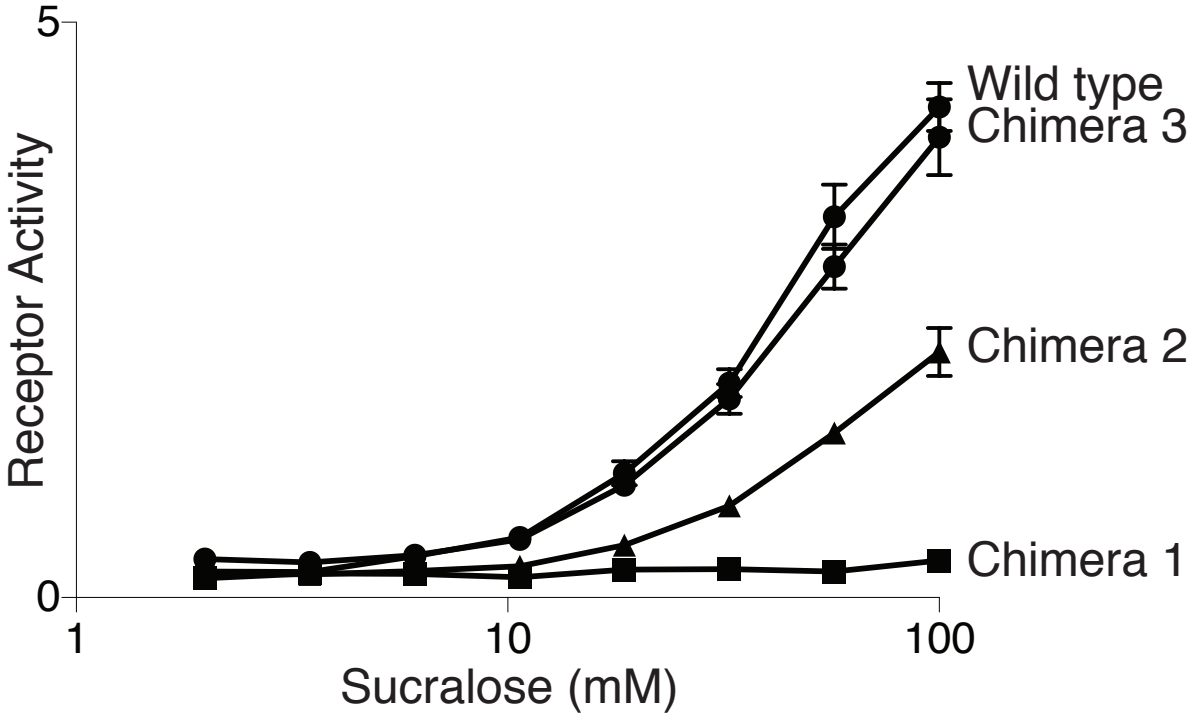

5
0
Receptor Activity
1
10
100
Sucralose (mM)
Wild type
Chimera 3
Chimera 2
Chimera 1

Figure S4

Fig. S4

Responses of chimeric T1R3s to sucralose. Responses of cells (n=6, mean ± SE) expressing wild type or chimeric T1R3 (Fig. 3A) and hummingbird T1R1 to different doses of sucralose.

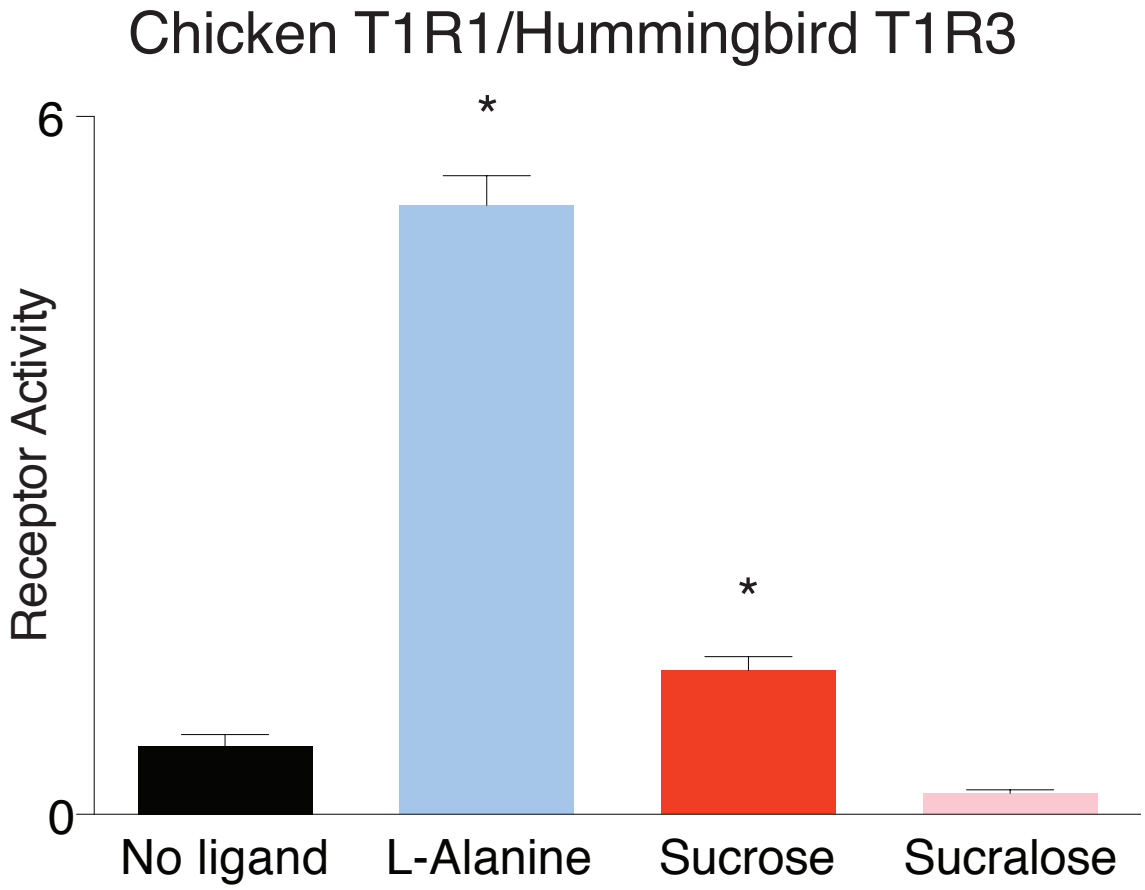

Chicken T1R1/Hummingbird T1R3
Receptor Activity
6
0
*
*
No ligand
L-Alanine
Sucrose
Sucralose

Figure S5

Fig. S5

Hummingbird T1R1 contributes to sugar responsiveness. Cells expressing chicken T1R1 and hummingbird T1R3 were analyzed for responses to L-alanine, sucrose, and sucralose (each 100 mM) using the cell-based assay (n=6, mean ± SE, *p≤0.05, Welch's *t*-test). Hummingbird T1R1 is also required for maximal responsiveness to sugar.

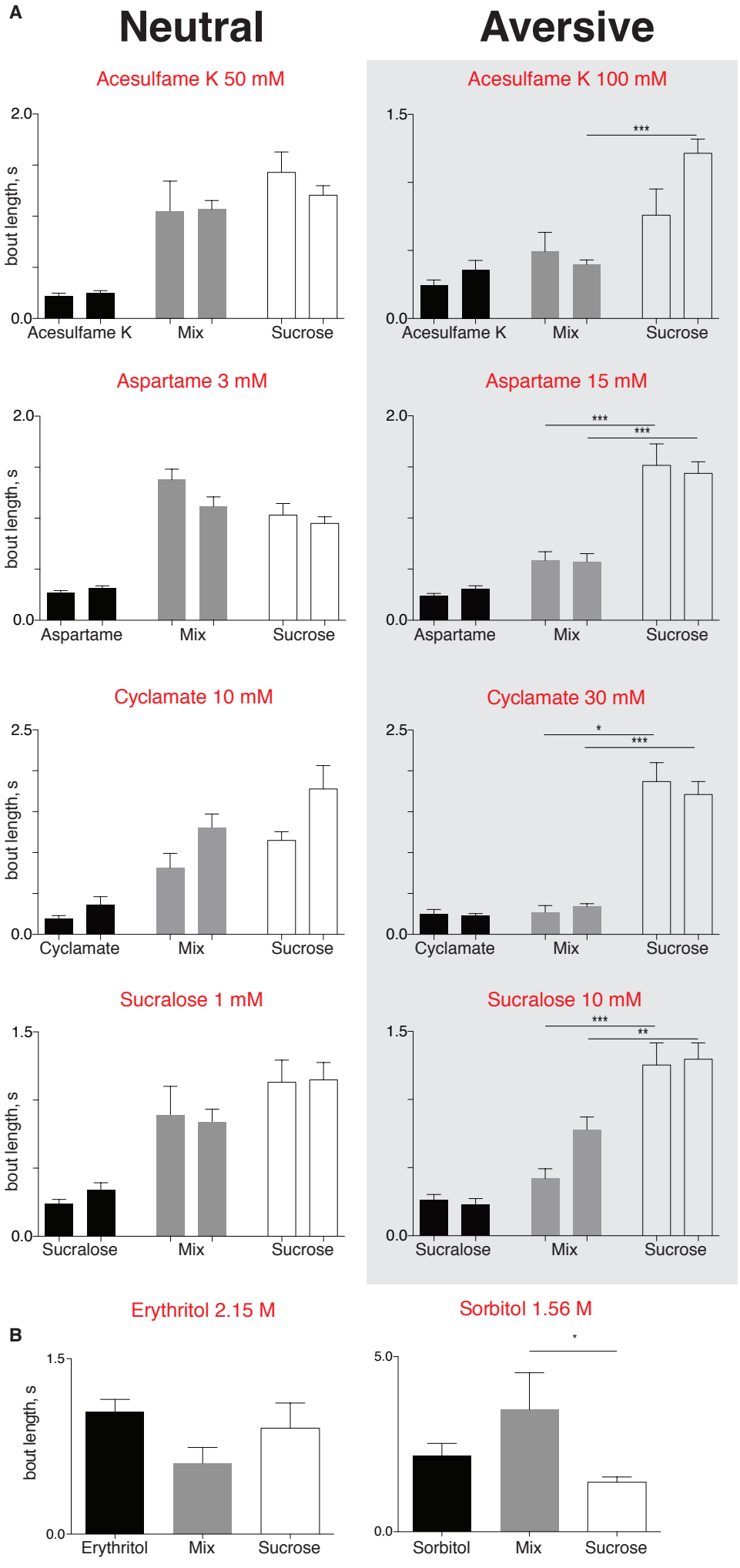

A
Neutral
Aversive
Acesulfame K 50 mM
Acesulfame K 100 mM
Aspartame 3 mM
Aspartame 15 mM
Cyclamate 10 mM
Cyclamate 30 mM
Sucralose 1 mM
Sucralose 10 mM
bout length, s
Acesulfame K
Aspartame
Cyclamate
Sucralose
Mix
Sucrose
B
Erythritol 2.15 M
Sorbitol 1.56 M
Erythritol
Sorbitol

Figure S6

Fig. S6

Synthetic sweeteners for humans are aversive to birds at high concentrations. (A) Response of wild hummingbirds to human sweeteners at low and high concentrations. Bout lengths of male Anna's hummingbirds (left bar) and all hummingbirds (right bar) to stimuli, sucrose (500 mM), and a mixture of stimuli and sucrose (500 mM) were determined (mean ± SE, sample sizes in Table S4, Kolmogorov-Smirnov tests to compare mixtures and sucrose solutions: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). **(B)** Responses of male Anna's hummingbirds to sugar alcohols, sucrose (500 mM) or mixtures of sugar alcohols and sucrose (500 mM).

Table S1. Accession numbers and genomic information for T1Rs. 62 T1R sequences were cloned, downloaded from Ensembl or GenBank, or extracted from whole genome sequences by BLAST searches. Species names (common and scientific), sequence source, download date, and accession number are listed. A subset of available fish T1R sequences were used for this analysis (*12, 40, 41, 42*).

Table S2. Analysis of positive selection in hummingbird, chicken, and swift T1Rs. (A) Models of evolution of T1Rs in specified lineages were compared with likelihood ratio tests using χ^2 tests of significance. The difference in model likelihoods (2*ΔlnL), degrees of freedom (*df*), and the critical value from the χ^2-distribution used to determine significant differences are presented. Model A v Model 1 is a test for positive selection in specified lineages, and Model A v Model A Null is an additional test that here does not rule out the possibility of relaxed selection. **(B)** Parameters of the CODEML models used in analyses of positive selection, including the number of free parameters (P), the proportion of sites (p_0 - p_{2b}) with the corresponding ω value, and numbers of positively selected sites from the Bayes Empirical Bayes (BEB) analysis. Sites in parenthesis indicate lineages which did not support Model A v Model 1.

Table S3. Description of sites in hummingbird T1Rs with evidence of positive selection. Residue number, amino acid identity, posterior probability, and information about the function of this site, or nearby sites in other family C GPCRs (*43, 44*).

Table S4. Sample sizes for behavioral assays involving wild hummingbirds. Numbers of visits of male Anna's hummingbirds (15 minute trial) or all birds (7 minutes or up to 200 total visits) for trials indicated, as reported in Figs. 4B and S6.

Movie S1. Hummingbirds discriminate between water and sucrose rapidly. High-speed video (slowed for viewing) of a ruby-throated hummingbird rejecting water presented in the top cuvette after three tongue licks (162 milliseconds). On average (5 birds, 2-3 trials per bird) rejections occurred within 3-4 tongue licks. Sucrose (500 mM, bottom cuvette) elicits a prolonged feeding bout.

Movie S2. Depiction of the behavioral assay involving wild hummingbirds. Video showing male Anna's hummingbirds (feeders 1 and 3) and a female hummingbird (feeder 6) in the behavioral paradigm. In this trial, feeders 1 and 4 contained aspartame (15 mM), feeders 2 and 5 contained a mixture of aspartame (15 mM) and sucrose (500 mM), and feeders 3 and 6 contained sucrose (500 mM). Feeders are numbered 1 through 6 in counter clockwise order. The bird at feeder 1 displayed a characteristic rejection behavior, while birds at feeders 3 and 6 fed for an extended duration.

Table S1: Accession numbers and genomic information for T1Rs

Species	Scientific name	Gene	Database	Download date	Accession number
Chicken	*Gallus gallus domesticus*	T1R1	cloned		KM091451 (GenBank)
		T1R3	cloned		KM091452 (GenBank)
Anna's hummingbird	*Calypte anna*	T1R1	cloned		KM091453 (GenBank)
		T1R3	cloned		KM091454 (GenBank)
Chimney swift	*Chaetura pelagica*	T1R1	cloned		KM091455 (GenBank)
		T1R3	cloned		KM091456 (GenBank)
Collared flycatcher	*Ficedula albicollis*	T1R1	Ensembl	11/1/13	ENSFALT00000010283
		T1R3	Genome	11/1/13	AGTO02.fsa.1/2/3
Mallard	*Anas platyrhynchos*	T1R1	Genome	11/1/13	ADON01.fsa.1/2/3
		T1R3	Genome	11/1/13	ADON01.fsa.1/2/3
Medium ground finch	*Geospiza fortis*	T1R1	Genome	11/1/13	AKZB01.fsa.1/2
		T1R3	GenBank	12/9/13	gi|543279702|ref|XM_005427699.1
Peregrine falcon	*Falco peregrinus*	T1R1	Genome	11/1/13	AKMT01.fsa.1/2
		T1R3	GenBank	12/9/13	gi|529436615|ref|XM_005238017.1
Puerto Rican amazon	*Amazona vittata*	T1R1	Genome	11/1/13	AOCU01.fsa.1/2
		T1R3	Genome	11/1/13	AOCU01.fsa.1/2
Rock dove	*Columba livia*	T1R1	Genome	11/1/13	AKCR01.fsa.1/2
		T1R3	Genome	11/1/13	AKCR01.fsa.1/2
Scarlet macaw	*Ara macao*	T1R1	Genome	11/1/13	AMXX01.fsa.1/2/3
		T1R3	Genome	11/1/13	AMXX01.fsa.1/2/3
Tibetan ground tit	*Pseudopodoces humilis*	T1R1	GenBank	10/9/13	gi|543370476|ref|XM_005528406.1
		T1R3	GenBank	12/9/13	gi|543374630|ref|XM_005530473.1
Wild turkey	*Meleagris gallopavo*	T1R1	Genome	11/1/13	ADDD01.fsa.1/2
		T1R3	Genome	11/1/13	ADDD01.fsa.1/2
Zebra finch	*Taeniopygia guttata*	T1R1	Genome	11/1/13	ABQF01.fsa.1/2/3/4
		T1R3	Genome	11/1/13	ABQF01.fsa.1/2/3/4
Chinese	*Alligator*	T1R1	Genome	11/1/13	AVPB01.fsa.1/2/3/4/5

alligator	*sinensis*				
		T1R2	GenBank	12/4/13	gi|557298567|ref|XM_006031490.1
		T1R3	GenBank	12/9/13	gi|557267013|ref|XM_006018839.1
Painted turtle	*Chrysemys picta bellii*	T1R1	GenBank	10/9/13	gi|530586781|ref|XM_005287086.1
		T1R2	GenBank	12/4/13	gi|530651565|ref|XM_005312106.1
		T1R3	GenBank	12/9/13	gi|530599183|ref|XM_005293033.1
Chinese softshell turtle	*Pelodiscus sinensis*	T1R1	Ensembl	12/9/13	ENSPSIT00000004761
		T1R2	GenBank	12/4/13	gi|558125603|ref|XM_006115098.1
		T1R3	Ensembl	12/9/13	ENSPSIT00000003074
Anole lizard	*Anolis carolinensis*	T1R1	Ensembl	10/31/13	ENSACAT00000011479
		T1R2	Ensembl	12/4/13	ENSACAT00000008692
		T1R3	Ensembl	12/9/13	ENSACAT00000013967
Opossum	*Monodelphis domestica*	T1R1	Ensembl	10/31/13	ENSMODT00000007553
		T1R2	Ensembl	12/4/13	ENSMODT00000026580
		T1R3	Ensembl	12/9/13	ENSMODT00000008007
Human	*Homo sapiens*	T1R1	Ensembl	10/31/13	ENST00000333172
		T1R2	Ensembl	12/4/13	ENST00000375371
		T1R3	Ensembl	12/9/13	ENST00000339381
Rat	*Rattus norvegicus*	T1R1	Ensembl	10/31/13	ENSRNOT00000013385
		T1R2	Ensembl	12/4/13	ENSRNOT00000025173
		T1R3	Ensembl	12/9/13	ENSRNOT00000026671
Dog	*Canis lupus familiaris*	T1R1	Ensembl	10/31/13	ENSCAFT00000031171
		T1R2	Ensembl	12/4/13	ENSCAFT00000024580
		T1R3	Ensembl	12/9/13	ENSCAFT00000030610
Pufferfish	*Takifugu rubripes*	T1R1	Ensembl	10/31/13	ENSTRUT00000040199
		T1R2	Ensembl	12/4/13	ENSTRUT00000038569
		T1R3	Ensembl	12/9/13	ENSTRUT00000038647
Stickleback	*Gasterosteus aculeatus*	T1R1	Ensembl	10/31/13	ENSGACT00000008412
		T1R3	Ensembl	12/9/13	ENSGACT00000010071

Medaka	*Oryzias latipes*	T1R1	Ensembl	10/31/13	ENSORLT00000004948
		T1R2	Ensembl	12/4/13	ENSORLT00000005451, ENSORLT00000005414, ENSORLT00000005495
		T1R3	Ensembl	12/9/13	ENSORLT00000005293
Zebrafish	*Danio rerio*	T1R1	Ensembl	10/31/13	ENSDART00000104214
		T1R2	Ensembl	12/4/13	ENSDART00000075125 ENSDART00000082509
		T1R3	Ensembl	12/9/13	ENSDART00000021369
Coelacanth	*Latimeria chalumnae*	T1R1	Ensembl	10/31/13	ENSLACT00000019188
		T1R2	GenBank	12/4/13	gi|556945589|ref|XM_005986117.1, gi|556945586|ref|XM_005986116.1
		T1R3	Ensembl	12/9/13	ENSLACT00000021918

Table S2: Analysis of positive selection in hummingbird, chicken, and swift T1Rs.

a) Likelihood ratio tests (LRT) comparing alternative models of evolution

Comparison	Likelihood of null model (lnL)	Likelihood of alternative model (lnL)	*df*	2*(ΔlnL)	Critical Value	Significant?
T1R3						
Hummingbird T1R3						
Model 1 v Model A	-46589.498	-46583.065	*2*	12.866	5.99	YES
Model A Null v Model A	-46583.708	-46583.065	*1*	1.286	3.84	NO
Swift T1R3						
Model 1 v Model A	-46589.498	-46586.767	*2*	5.460	5.99	NO
Chicken T1R3						
Model 1 v Model A	-46589.498	-46589.498	*2*	0	5.99	NO
T1R1						
Hummingbird T1R1						
Model 1 v Model A	-42448.130	-42444.458	2	7.344	5.99	YES
Model A Null v Model A	-42444.867	-42444.458	1	0.818	3.84	NO
Swift T1R1						
Model 1 v Model A	-42448.130	-42445.464	2	5.331	5.99	NO
Chicken T1R1						
Model 1 v Model A (ω_i=2)	-42448.130	-42445.990	2	4.280	5.99	NO

b) Parameter estimates for CODEML analyses

Model	P	Estimates of parameters	Positively selected sites
T1R3			
M1:Neutral	2	$p_0 = 0.670$, $p_1 = 0.330$, $\omega_0 = 0.163$, $\omega_1 = 1$	Not allowed
Branch-specific: hummingbird T1R3			
Model A	4	$p_0 = 0.629$, $p_1 = 0.309$ $p_{2a} = 0.041$, $p_{2b} = 0.020$, $\omega_0 = 0.162$ $\omega_1 = 1$, $\omega_2 = 2.42$	BEB 6 > 0.5 (1 > 0.95)
Model A null	3	$p_0 = 0.586$, $p_1 = 0.289$, $p_{2a} = 0.084$, $p_{2b} = 0.041$, $\omega_0 = 0.161$, $\omega_1 = 1$, $\omega_2 = 1$	Not allowed
Branch-specific: swift T1R3			
Model A	4	$p_0 = 0.662$, $p_1 = 0.325$ $p_{2a} = 0.009$, $p_{2b} = 0.004$, $\omega_0 = 0.163$ $\omega_1 = 1$, $\omega_2 = 6.957$	(BEB 3 > 0.5)
Branch-specific: chicken T1R3			
Model A	4	$p_0 = 0.670$, $p_1 = 0.330$ $p_{2a} = 0$, $p_{2b} = 0$, $\omega_0 = 0.163$ $\omega_1 = 1$, $\omega_2 = 1$	None
T1R1			
M1:Neutral	2	$p_0 = 0.651$, $p_1 = 0.349$, $\omega_0 = 0.147$, $\omega_1 = 1$	Not allowed
Branch-specific: hummingbird T1R1			
Model A	4	$p_0 = 0.639$, $p_1 = 0.341$ $p_{2a} = 0.013$, $p_{2b} = 0.007$, $\omega_0 = 0.146$ $\omega_1 = 1$, $\omega_2 = 6.362$	BEB 6 > 0.5
Model A null	3	$p_0 = 0.596$, $p_1 = 0.318$, $p_{2a} = 0.056$, $p_{2b} = 0.030$, $\omega_0 = 0.145$, $\omega_1 = 1$, $\omega_2 = 1$	Not allowed
Branch-specific: swift T1R1			
Model A	4	$p_0 = 0.647$, $p_1 = 0.345$ $p_{2a} = 0.005$, $p_{2b} = 0.003$, $\omega_0 = 0.147$ $\omega_1 = 1$, $\omega_2 = 19.707$	(BEB 4 > 0.5)
Branch-specific: chicken T1R1			
Model A (ω_i=2)	4	$p_0 = 0.644$, $p_1 = 0.343$ $p_{2a} = 0.009$, $p_{2b} = 0.005$, $\omega_0 = 0.146$, $\omega_1 = 1$, $\omega_2 = 15.349$	(BEB 5 > 0.5)

ω_i= initial starting value of ω, if a better fit than (ω_i=0), assessed via likelihood ratio tests

Table S3: Description of sites in hummingbird T1Rs with evidence of positive selection.

a) T1R3

Residue #	Amino acid	BEB posterior probability	Functional information regarding site
206	I	0.694	Important residue for the hummingbird sugar response (this chimeric analysis)
237	S	0.571	Important residue for the hummingbird sugar response (this chimeric analysis)
372	A	0.593	Aligns to S362 of hummingbird T1R1 (Table S3b); 4 residues away from *Sac* phenotype residue 371 (43)
384	L	0.641	Aligns to human T1R2 residue 383, important for sucrose and sucralose ligand binding (44)
511	Y	0.804	
530	Q	0.985	

b) T1R1

Residue #	Amino acid	BEB posterior probability	Functional information regarding site
54	Y	0.795	1 residue away from human T1R1 residue 71 (important for ribonucelotide-potentiated umami response) (21) and 1 residue away from human T1R2 residue 65 (involved in sucralose binding) (44)
58	A	0.858	3 residues away from human T1R1 residue 71 (important for ribonucelotide-potentiated umami response) (21)
362	S	0.702	Aligns to A372 identified in hummingbird T1R3 analysis (Table S3a); 4 residues away from *Sac* phenotype residue 371 (43)
430	G	0.852	
449	S	0.520	1 residue away from human T1R1 residue 460, important for broad tuning of umami receptor (18)
580	F	0.773	

Table S4: Sample sizes for behavioral assays involving wild hummingbirds

a) carbohydrate trials and sugar alcohols

	Number of visits		
Stimulus	# visits of Anna's males in 15 minute trial		
	1	**2**	**3**
1 = Water, 2 = Sucrose 250 mM, 3 = Sucrose 500 mM	12	14	67
1 = Glucose 1M, 2 = Fructose 1M, 3 = Sucrose 1M	19	70	16
Erythritol 2.15 M: **1 = Stimulus, 2 = Mix, 3 = Sucrose**	37	14	8
Sorbitol 1.56 M **1 = Stimulus, 2 = Mix, 3 = Sucrose**	16	23	46

b) synthetic human sweeteners

	Number of visits					
Stimulus	# visits of Anna's males in 15 minute trial			# visits of all birds (first rotation only)		
	Stimulus	Mix	Sucrose	Stimulus	Mix	Sucrose
Aspartame 3 mM*	21	79	42	19	77	104
Aspartame 15 mM	10	50	26	12	53	122
Acesulfame K 50 mM *	10	16	39	30	85	86
Acesulfame K 100 mM	11	10	20	6	16	95
Cyclamate 10 mM	7	11	55	10	43	38
Cyclamate 30 mM	3	2	17	27	42	114
Sucralose 1 mM*	22	10	32	25	76	101
Sucralose 10 mM	13	24	39	7	56	65

* for all-bird trial, first ~200 bouts scored

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