# Evolution of sweet taste perception in hummingbirds

**Evolution of sweet taste perception in hummingbirds**

A dissertation presented

by

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## Abstract

Mammals have three members of the small taste receptor gene family responsible for the perception of sweet and savory tastes: two genes (*T1R2* and *T1R3*) comprise the canonical sweet receptor, and a third gene, *T1R1*, acts with *T1R3* to make the savory receptor. Here, in a joint effort with a team of international collaborators, we show that even though birds are missing the taste receptor gene (*T1R2*) required by other vertebrates to perceive carbohydrates and sweeteners, hummingbirds still detect sugars—but in a novel way. This project spanned multiple fields and field sites, integrating taste tests on wild birds, behavioral analysis of captive animals, bioinformatics, receptor cloning, and cell-based functional assays.

The first published avian genome, that of the chicken, revealed a surprising lack of *T1R2*. Chickens are sweet-insensitive: however, many nectar-feeding birds appear highly attuned to sugars like sucrose, fructose and glucose. Our initial field experiments with a panel of artificial sweeteners as well as high-speed filming and choice tests on captive birds indicated a rapid response to sugars rather than a post-ingestive metabolic sensing of caloric value. As the response appeared sensory, we pursued a candidate gene approach to search for possible taste receptors, and cloned *T1R* taste receptors from chickens, hummingbirds, and swifts. By analyzing genomes from an additional 10 birds and an alligator, we documented widespread absence of *T1R2* and identified signatures of positive selection in the remaining hummingbird *T1R*s.

Together with Dr. Yasuka Toda at the University of Tokyo, we were able to test the function of these receptors in cell culture. We used a cell-based luminescence assay to measure functional responses. As expected, chicken and swift receptors responded to amino acids, but, surprisingly, the umami receptor in hummingbirds had acquired a new function and was now sensitive to carbohydrates as well. Chimeric studies of receptors containing hummingbird and chicken sequence identified 19 mutations involved in this functional change: since divergence from swifts, the umami receptor underwent extensive re-modeling. Further behavioral tests with wild hummingbirds revealed that most agonists from the cell-based assay were appetitive, while artificial sweeteners which did not activate the receptors were not preferred—a concordance between *in vivo* and *in vitro* studies that indicates that this re-purposed receptor guides hummingbird taste behavior.

Diet shifts have profound physiological effects and evolutionary ramifications: the radiation of hummingbirds is likely due, at least in part, to their ability to colonize an empty niche. However, much remains to be learned about the roles of taste in changes in diet, and the causes and effects of shifts in diet and perception are often unclear. For instance, birds appear to have lost *T1R2* early in their evolutionary history. As they are the descendants of carnivorous dinosaurs, birds may have experienced relaxed selection on the sweet receptor similar to that seen in mammalian carnivores; alternatively, the loss could be due to the overall genome-wide reduction seen in birds. In Chapter 3, we begin to investigate causes and consequences of the changes in hummingbird taste receptors, and present new behavioral studies regarding the ability to detect amino acids. Together, these findings raise questions about the evolution of sensory systems and of neural circuits underlying perception: studying taste receptors in a comparative context yields insight into basic aspects of the evolutionary process as well as molecular mechanisms underlying behavior.

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**Humming-bird** ***by D. H. Lawrence***

I can imagine, in some otherworld

Primeval-dumb, far back

In that most awful stillness, that only gasped and hummed,

Humming-birds raced down the avenues.

Before anything had a soul,

While life was a heave of Matter, half inanimate,

This little bit chipped off in brilliance

And went whizzing through the slow, vast, succulent stems.

I believe there were no flowers then,

In the world where humming-birds flashed ahead of creation.

I believe he pierced the slow vegetable veins with his long beak.

Probably he was big

As mosses, and little lizards, they say, were once big.

Probably he was a jabbing, terrifying monster.

We look at him through the wrong end of the long telescope of Time,

Luckily for us.

I dedicate this dissertation to my wonderful family,

and to the memory of my grandfather,

John W. Baldwin.

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# Introduction

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Sensory systems define how we experience the world. Each living thing exists in its own unique perceived environment or '*Umwelt*'—a German word signifying merely 'environment' today but adopted by Jacob von Uexküll in the 19th century to refer to this concept of a private sensory world constructed by each individual organism. As stimuli relevant for one organism are not always important for another to detect, these systems adapt and evolve and often differ dramatically between species. Yet sensory systems are also the product of an organism's evolutionary history, as well as a reflection of the pressures of the present.

Hummingbirds and their taste receptors represent a clear example of this complex suit of influences, and by studying their taste system, our conception of the meaning of 'sweetness' and how we assume it must be detected has been changed (*1*). As is true for all organisms, birds' sensory systems are molded not only by the current environment but also by the demands of past worlds and by selection acting on other aspects of the phenotype. Genetic drift and demographic factors that shape every genome play a part in shaping sensory systems as well. To fully understand the role of sweet taste perception and why or how it initially arose, it is necessary to go back in evolutionary history to lampreys and the first appearance of vertebrates, but even here, surprisingly little is known for certain.

## Chemosensory systems and the evolutionary process

Vertebrates detect many different types of sensory stimuli. Mechanical forces are detected by specialized hair cells and touch receptors, and electromagnetic information, including magnetic fields, the broad spectrum from infrared radiation to visible and ultraviolet light as well as passively and actively generated electrical fields, is sensed by other specialized end-organs and classes of receptors (for example, opsins (*2*) and TRP channels (*3*)). A lot goes on outside of our

ability to detect it: pit vipers 'see' heat (*4*), bats echolocate (*5*), a hunting platypus detects muscle twitches (*6*). Glass knifefish and ghost knifefish (*7*), socially electrogenic, generate and sense the electric fields of their conspecifics. Remote sensing and satellite technology now analyze the electromagnetic spectrum of images taken from space to determine species composition of forests (*8*). There are many channels of information to tune into.

Chemicals also provide information about the external world, and through olfaction (*9*), taste (*10*), and in some species, a structure that detects pheromones called the vomeronasal organ, vertebrates detect environmental and social cues (*11*). The senses are used in different behavioral contexts such as foraging and mate choice, but on the receptor level, they work in similar ways. Many chemosensory receptors are proteins called G-Protein Coupled Receptors, or GPCRS (*12*). GPCRS are the largest superfamily of genes in vertebrate genomes, consisting of over a thousand members in many mammals. Sensory GPCRs are receptors that span the membrane of sensory cells, present, for instance, in the olfactory sensory neurons that line the nasal epithelium and in taste cells that comprise the onion-shaped taste buds in the oral cavity. When the receptors detect ligands in the extracellular space, they undergo a conformational change and initiate an intracellular cascade of signaling events. This eventually leads to neurotransmitter release and neuronal activity: information from these cells is then sent centrally to the brain. Other chemicals in food act via different, non-taste pathways. Peppers are often perceived as hot—in mammals, pain receptors and temperature sensors are tricked by chemicals to produce the heat of capsaicin and the coolness of wintergreen (*13*).

Gene duplication is a fundamental mechanism by which genomic and organismic complexity is generated (*14*). Many chemosensory receptors are members of large gene families, the product of gene duplication events. These receptor families are notoriously dynamic: they

expand and contract across the vertebrate tree, allowing us to investigate basic aspects of evolutionary change on a molecular level, such as rates of gene birth and death and phenomena like pseudogenization and gene convergence. Often changes in gene number are related to niche: mice are highly olfactory, for instance, and have far more olfactory receptors than humans. Yet the correlation with ecological drivers is not always apparent. Neutral factors or 'genomic drift' can also shape the size of receptor repertoires (*15*).

Because of their role in directly sensing the external environment, chemosensory receptors provide a direct link between stimulus detection and behavior. Slight changes in these receptors can render a stimulus imperceptible and thus can have an immediate impact on an organism's actions: the connection between genotype and phenotype is very clear. This direct link between a gene and a measurable phenotype—here, a behavior—makes sensory receptors tractable model systems for the study of basic evolutionary processes. In addition to dynamics of gene duplication, the effect of individual mutations can be studied. The magnitude of the effect of a single mutation, the importance of the order in which mutations occur, and the extent of epistasis and interactions between sites can all be dissected. Evolution happens because of changes to individual bases pairs; sensory receptors allow us to examine aspects of this process in a simplified form.

The gustatory system and the *T1R* gene family

The taste system in vertebrates is far less complex than the olfactory system. Taste mediates basic, instinctive foraging behaviors closely linked with survival: toxic compounds in foods are rapidly sensed and rejected before they are ingested: we spit out spoiled food now to save us from vomiting later. Appetitive tastes alert organisms of nutritionally valuable resources.

If something is tasty, there's a good chance it's nutritious—at least before fast food. Our innate tastes are modulated by a host of additional factors; appetites change in accordance with physiological needs and can vary over daily and seasonal timescales. Learning modifies preferences: adults may crave coffee, cigarettes, tea or beer, all of which contain bitter ligands babies reject.

The basic tastes and the receptors that underlie them are relatively conserved across vertebrates, from fish through reptiles. Invertebrate chemosensory receptors detect similar chemicals but have independently evolved--they are not homologous and share no sequence similarity, a clear case of convergent evolution (*10*, *16*). New research continues to prove the existence of new tastes and to elucidate the mechanisms behind them, and the number of these non-canonical tastes varies across species. Flies (*17*), frogs (*18*) and maybe mammals (*19*) can taste water by separate pathways. A recently discovered mechanism allows carbonation to be sensed (*20*). Fats are detected not only by their tactile oiliness but also by chemosensory g-protein coupled receptors (GPCRS) as well (*21*). These new tastes aside, there are 5 generally accepted basic tastes shared by humans and many other mammals: salty and sour are detected by ion channels, and the other three tastes, bitter, sweet and umami (from the word for savory in Japanese) are detected by GPCRS. Two main families exist: bitter taste receptors make up one family, called the Type 2 taste receptors, or *T2R*s. *T2R*s are composed of a single exon coding for a trans-membrane region; these genes are phylogenetically related to odorant receptors. Bitter receptors detect many varied ligands like toxins, many of which are plant metabolites such as nicotine and caffeine, yet these diverse stimuli all result in a common innate behavioral output: aversion. Bitter receptors are expressed in specific cells in taste buds, and are often co-expressed with each other, but not with the receptors for other tastes (*22*).

Both of the other two tastes, sweet and umami, are pleasurable and appetitive. They are detected by receptors from a second taste receptor gene family, called the *T1R*s, which is unusually small in number: most vertebrates appear to have only three members (*23*). The functional taste receptors, in their presumed natural state in taste buds, are heterodimers, meaning that they are composed of two different proteins from this family. In the case of umami, T1R3 interacts with T1R1 (*24*), and for the sweet receptor, T1R3 interacts with T1R2 (*25*) (Figure 1). Thus, each functional, appetitive taste receptor is composed of a unique and a shared component. These three genes are different in structure from the bitter taste receptors; they are large, are composed of multiple exons, and have a long domain that extends outside the cell. They are part of the Family 3 Class C GPCRs (*26*), distantly related to a family of pheromone receptors as well as to receptors which sense amino acids used in neurotransmission, such as the metabotropic glutamate receptor, for which a crystal structure has been solved (*27*).

Of the two appetitive tastes, umami is less well-known; it was discovered only in 1908 by a Japanese researcher distilling seaweed (*28*). Umami is the taste of the amino acids that make up proteins, and requires T1R1: knock-out mice lacking T1R1 are umami-insensitive. In some primates, including humans and close relatives, the receptor is narrowly tuned and responds most strongly to glutamic acid (known to cooks as MSG), as well as to another acidic amino acid, aspartic acid. In other primates, in mice and in fish, these receptors are more broadly tuned, however, and respond to a wider range of L-amino acids (*24*, *29*, *30*). The dietary reasons behind the receptor tuning is unclear, as most essential amino acids (those that cannot be internally synthesized but must be ingested) are *not* among those preferred by the receptor. Perhaps one amino acid signals presence of the rest.

The sweet receptor is also made up of T1R3, but now acting in combination with T1R2. Anything we call sweet activates this receptor, including carbohydrates, artificial sweeteners, and certain D-amino acids. T1R2 is necessary: knock-out mice lacking T1R2 are insensitive to all sweet compounds (*31*). While mammals prefer the same carbohydrates, other sweeteners do not always work across species (*32*): most mice do not detect aspartame (*33*), the artificial sweetener in Diet Coke, as well as certain intensely sweet proteins such as brazzein and thaumatin, plant-derived compounds that activate the taste receptors of primates (*34*).

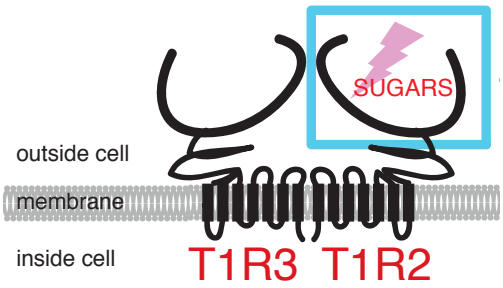


**Figure 1. Schematic of the sweet receptor heterodimer.** The "venus flytrap" domain of *T1R2* is shown in blue.

Much of our understanding of taste is new: these receptors were discovered little over a decade ago. The exact location where specific chemicals like sugars or sweeteners interact with the receptor is not yet fully elucidated: to date, a crystal structure is only available for the metabotropic glutamate receptor, mGluR, also a Class C receptor (*27*). From similarities between these proteins, it is clear that each long extracellular region—called the venus flytrap region because of resemblance to the carnivorous plant—is composed of two lobes and an internal cleft. In T1R2, this extracellular region is thought to be the primary interaction site for carbohydrates with the receptor, but mapping and mutagenesis studies reveal that many sweeteners and sweet taste enhancers (*35*) interact with regions outside of the traditional ligand-binding cleft: for

instance, a sweetener called cyclamate is thought to interact with the transmembrane region of T1R3 (*36*), and in biochemical assays, the venus flytrap domain of both T1R2 as wells as T1R3 can bind to carbohydrates (*37*). Many other questions around the precise role and mechanism of activation of these genes await explanation and study.

The origin of the taste system in vertebrates

Biological questions can be tackled from a number of different angles. Mechanistic studies of receptor activation elucidate some aspects of the biology of taste, but other approaches tell us different stories. Clearly understanding our sense of taste requires us to probe the evolutionary origin of this sensation: how did we, as vertebrates, develop the sensory organs in our mouths that respond to five tastes, our gustatory toolkit? Why do we need to be attuned to the taste of sugars, and when did this need first arise? Looking into our evolutionary history provides clues to the origin and importance of our mammalian tastes, yet many aspects of the sensation of sweetness are still a mystery.

Taste buds, as we know them, first appear in our earliest vertebrate ancestors, the lampreys (*38*)—the jawless, cartilaginous fish which arose after our earlier diverging relatives, the sea-squirts or tunicates, and the related amphioxus. In lampreys, the pharynx seen in amphioxus is no longer flanked by a ciliated opening or mouth but is a circular structure, ringed by concentric teeth, with which lampreys latch onto flanks of other fish (Figure 2). Once attached, the lamprey scrapes away at the host's flesh with a peculiar rasping "tongue," a structure not homologous to tongues of any other vertebrate (*39*). In other vertebrates, the tongue is supported by a bony structure called the hyoid apparatus, developed from the second branchial

arch (the first arch forms vertebrate jaws); in lampreys the tongue, also lined with tooth rows, is an independently-derived piston.

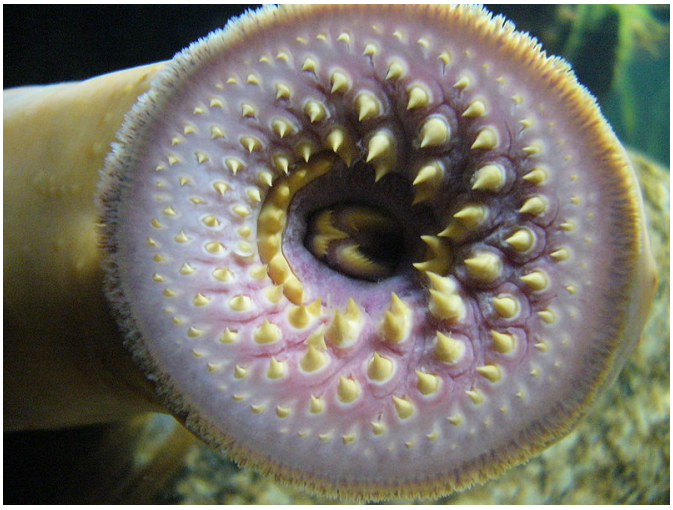

**Figure 2. Sea lamprey mouth (***Petromyzon marinus*).(*Photo: Drow male, Wikimedia commons*).

While lampreys lack a true tongue, they do possess tiny taste buds which then remain, conserved in form, throughout the vertebrate trajectory. Cells expressing the sweet taste receptor are found together with cells expressing receptors for the other basic tastes, forming clusters of 20-100 cells called taste buds. In most vertebrates, from lampreys onwards, taste buds share a similar cellular organization: buds are embedded in the oral epithelium (the tongue, the palate, the pharynx) or in some teleost fish, also occur extra-orally on the barbels and surface of the body (*38*). This is another way in which our Umwelt differs—think of this when you swim.

Clusters of taste buds are called papillae. Mammals have different types of papillae: large circumvallate papillae are found at the back of the tongue, oblong foliate papillae are located on the sides of the tongue, and smaller fungiform papillae occur on the tongue's surface. However, not all vertebrates share this organization. After metamorphosis, some adult frogs develop flat

structures called taste disks (*38*). In birds the large, circumvallate papillae are lacking, and most buds occur in isolation or as part of fungiform-like papillae. The small size of fungiform buds and the bony keratinous nature of the avian tongue made taste bud identification more difficult in birds than in other vertebrate groups: it wasn't until the early 1900s that anyone described these structures in birds (*40*, *41*). Once identified, they were found, though sparse in number, in all species examined, although often only the tongue base was sectioned. Since taste buds appear on the palate in many birds, reliable estimates of total number are lacking for most species, but it is thought that chickens have around 300 buds (*42*)--around two to three orders of magnitude fewer taste buds than in many mammals (*43*).

As in mammals, taste buds in birds are innervated by different cranial nerves, depending on the location in the mouth. The facial nerve (cranial nerve VII) innervates buds in the lower beak and top palate, the glossopharyngeal (IX) innervates buds in portions of the tongue (*44-46*). In mice, and perhaps in birds as well, the information from different taste cells (bitter, sweet, salty, umami) is processed as separate "labeled lines" (*47*, *48*); these qualities remain distinct even in the gustatory cortex. It is the cell that encodes the quality of the tastant: if sweet-detecting cells are engineered to express a synthetic receptor, then the new ligand for that receptor becomes a preferred stimulus (*31*). External reality stops at the taste cell: afterwards, it becomes a neural signal.

Natural loss of T1Rs correlated with diets

Unlike most other chemosensory gene families, the *T1R* gene family is small, and outside of fish, there are no lineage-specific duplications: most vertebrates have only three members (*49*). This is in stark contrast to other receptor families such as odorant receptors (ORs), trace-amine

associated receptors (TAARs), bitter taste receptors (T2Rs) and even the phylogenetically-related Family 3 Class C pheromone receptors (V2Rs) (*15*) and may indicate an as-yet unidentified constraint. T1Rs are expressed in other tissues outside of the oral cavity—in the gut, the pancreas, and the testes, for instance (*50*, *51*). While the functional roles in these other tissues are not yet entirely clear, the pattern is not unprecedented: sperm express olfactory receptors as well (*52*). Perhaps expression in other tissues impacts the evolutionary dynamics of this family.

Even though *T1R* duplication is rare in tetrapods, a number of instances of gene loss have been documented in this family, and strong evidence links the lack of these genes with the diet of an organism. Pandas, for instance, have a non-functional, or pseudogenized *T1R1* (*53*), and this pseudogenization event has been estimated to have occurred at approximately the same time that pandas switched their diet from meat to bamboo (*54*). In cats, *T1R2*, the unique part of the sweet heterodimer, has been pseudogenized—rendering cats insensitive to sucrose (*55*). The loss-of-function mutations in *T1R2* that prevent this gene from being expressed in taste buds are present in cats' close relatives, lions and tigers and cheetahs, suggesting a connection with a carnivorous lifestyle. Later research found the *T1R2* gene to be independently pseudogenized in other mammalian carnivores by different mutations, strengthening this connection (*56*). However, causality is unclear: whether the receptor was lost prior to a change in diet, or only subsequently, is not known.

Some other mysteries remain: many aquatic organisms such as whales (*57*) and seals (*58*) appear to have a degenerated taste system and may be lacking functional *T1R*s. Vampire bats have lost *T1R2* (*59*), perhaps related to the blood they drink or maybe simply to their limited diet—they also display a widespread loss of bitter receptors (*60*). Interestingly, the umami receptor of all bats is also non-functional, sparking speculation regarding the function of the taste

system in bats more broadly (*61*). While seemingly simple, the relationship between taste and diet can in cases be complex (*62*).

The tastes of chicken

After the chicken genome was published in 2004 (*63*), researchers discovered that *T1R2*, as in cats, was missing from the genome (*23*, *64*): but, unlike in felines, it was entirely absent and not even detected as a pseudogene. Did other birds also lack this gene, or was it simply an anomalous loss only in chickens? Subsequently, the zebra finch and turkey genomes were published and also showed no sign of this gene. The lack of even a remnant of the gene seemed consistent with a single, early loss: the order of the genes flanking *T1R2* is conserved in chicken but no signature of *T1R2* was recognizable in the intervening 16,000 bases. If birds were missing *T1R2* due to a single, early event —perhaps due to the carnivorous diet of their theropod dinosaur ancestors — a puzzle remained: how are nectar-feeding birds detecting sugars?

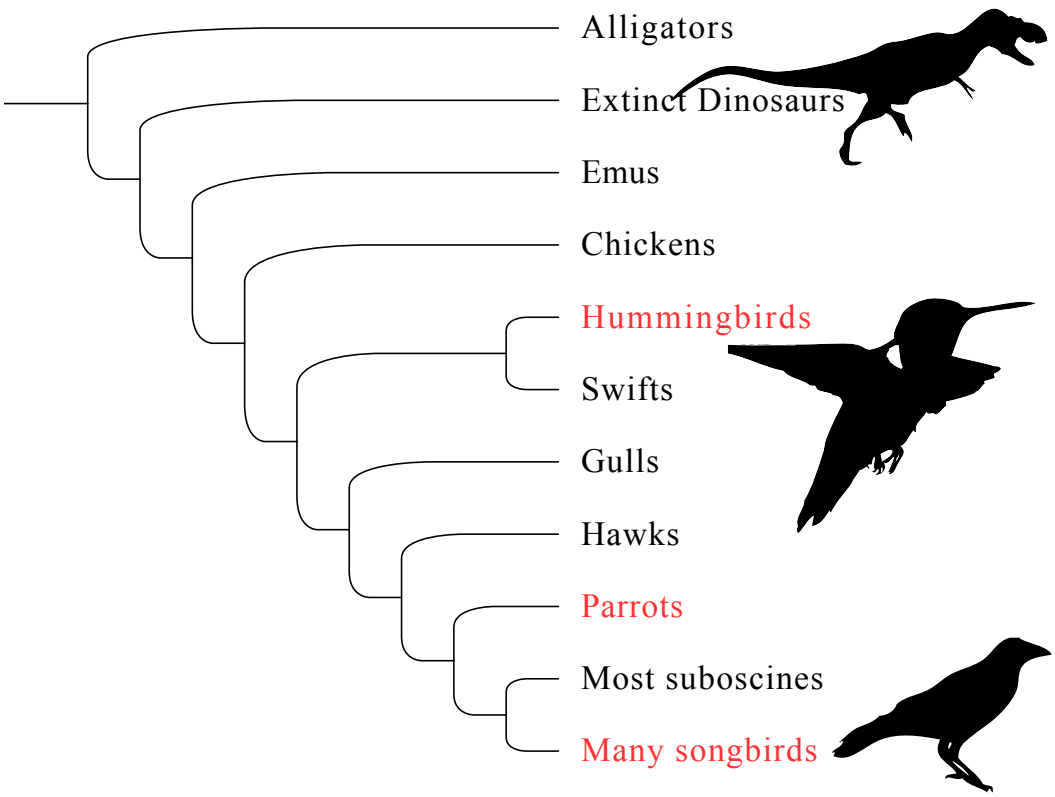


**Figure 3. Schematic of the evolutionary tree of birds**. Selected independent evolutions of nectar-feeding are shown in red.

Nectarivory, diet types, and taste preferences in birds

With over 10,000 species, birds, as a group, exhibit extreme behavioral, morphological, and dietary diversity: they look different, act differently, and eat different things. Granivores eat seeds, other species eat mammal-meat, fish, or insects. Others are omnivores and consume animals and plants. Dietary niches can be broad or narrow, phylogenetically widespread or unique: for instance, the Amazonian taxonomically enigmatic hoatzin convergently evolved the ability to ferment plant matter in a modified foregut, like a cow, and thus can sustain itself on a diet of leaves (*65*). Many birds have also coevolved with plants and are important seed and pollen dispersers; fruit-eaters (frugivores) often eat the fleshy outer covering, depositing the

seeds miles away (*66*). Multiple groups of birds have independently evolved nectar-feeding lifestyles, obtaining nutrition from the carbohydrate reserves in nectar, as well as catching supplemental insects to obtain sufficient nitrogen. Not all nectar-feeders are close relatives (Figure 3); many songbirds are nectarivorous (sunbirds, as well as the well-named honeyeaters and honeycreepers, flowerpeckers and flowerpiercers) (*67*). Other nectar-feeding groups include certain parrots, called lorikeets, as well one small group of suboscines (the sister group to songbirds) called asities (*68*), which live in Madagascar—all of which are evolutionarily quite distinct from the well-known, specialized, species-rich group of hummingbirds. The natural history of all the nectar-feeders seems to strongly indicate that they can detect and prefer carbohydrates, in a manner similar to most mammals. In fact, the ability of birds as a whole to detect and innately prefer sugars has never been explicitly questioned. Umwelt-empathy is not our default mode of operation.

One answer to how birds lacking *T1R2* still sense sugars could be that birds may not actually be responding to the *taste* of nectar: flowers are multi-modal stimuli and could be attractive for multiple reasons, such as the presence of visual or olfactory cues. Nectar, while primarily composed of the "big three" nectar sugars (sucrose, glucose, and fructose) is a complex mixture: depending on the species, it can contain varying mixtures of amino acids (*69*) or even multiple plant metabolites, such as nicotine (*70*). Alternatively, sugars might be rapidly metabolized, and hummingbirds' attraction to carbohydrate-rich nectar could be a post-ingestive response to caloric value, similar perhaps to a 'sugar buzz', as opposed to a sensory response communicated by the taste bud alone. Interpreting sensory abilities of fruit-eaters from natural history is even more difficult (*71*): fruits are complex, and other aspects of their taste or smell could lure in bird dispersers.

Evolution of nectarivory in hummingbirds

The ancestry of hummingbirds makes their sweet-tasting abilities particularly fascinating. Hummingbirds are unequivocally related to swifts—fast, agile, swallow-like birds with oblong bodies and boomerang-shaped wings—as well as to other members of a clade called Caprimulgiformes. Together with swifts and hummingbirds, this group is now collectively referred to as the Strisores (*72*), and contains cryptically-colored, nocturnal species—potoos, oilbirds, frogmouths, owlet-nightjars as well as the 'goatsuckers', the nighthawks and nightjars from which the original name stemmed. Most of these species (with the exception of the bizarre cave-dwelling, palm-fruit eating, echolocating oilbird) are specialized insectivores, subsisting solely on insects caught on the wing. Most are also active in darkness, either wholly nocturnal or crepuscular, hunting in the half-light of predawn or early evening. Habits of day-activity, or diurnality, evolved in the lineage leading to hummingbirds and swifts, and the fossil record indicates that the common ancestor of this group was likely swift-like in morphology (*73*). Thus, the ancestor of hummingbirds likely had no interest in flowers and their difficult, narrow corollas, but was a bird that spent most of its life high above the canopy, inhabiting a buzzing, swarming, acrobatic Umwelt, bent on snatching tiny flying prey.

**Figure 4. A wild male Anna's hummingbird (*Calypte anna*) in a feeding trial** (*Photo credit: Maude Baldwin*)

The order of events by which an aerial insectivore such as a swift dramatically changed in overall body size, wing shape, bill morphology, digestive physiology and behavior is a matter of speculation and open discussion today, so many millions of years later, but a recent fossil showing a "stem" swift with less elongated wings but nevertheless a swift-like bill supports a hypothesis that hovering capability may have evolved first (*74*). Perhaps the ancestors of hummingbirds were gleaning insects off flowers and gradually became more associated with these flowers and the nectar secreted to entice flies and bees. A small change in the bird's receptor activity could have perhaps allowed for the gain of a slight ability to detect the available carbohydrate resources, leading to increased selection pressure on changes in receptor function: once selection takes hold, the effect can be reinforced. While the divergence dates of hummingbirds and swifts are debated (some molecular estimates yield dates of ~70 mya, far too old to be probable (*75*)), it appears likely, from the fossil record, that the split may have happened ~50 mya, in the Eocene. At this time, flowering plants were abundant and their nectar

content may have been shaped by the sugar preferences of their invertebrate pollinators. Avian nectarivory may have not arisen yet in any group, leaving the niche wide open for exploitation: a newly-described fossil of a songbird relative from the Eocene (47 million years ago) with pollen grains in the stomach is the earliest evidence for bird nectar-feeding behavior to date (*76*).

Hummingbirds, today, are very varied and widespread—but only occur in the Western hemisphere. Surprisingly, however, the earliest fossil hummingbird is from Europe. It dates to the Oligocene, around 30 million years ago: presumably, hummingbirds' ranges must have historically been far broader (*77*, *78*). Gardeners from France claiming to have seen tiny avian flower visitors are likely to have been fooled by hawkmoths—indeed, the often striking similarity between locomotion and color patterns triggers us to wonder, millions of years later: did moths evolve to mimic birds? Or vice versa?

The center of diversity of living hummingbirds is Central and South America. Some species make it farther north, but not many. The United States is particular species-poor: the East Coast of the United States has a single species, the ruby-throated hummingbird, while sites in California boast six: many xeric, desert-loving species, as well as migrants such as the rufous hummingbird, which breed in temperate coastal rainforests farther up the coast into Alaska. Farther south, however, numbers skyrocket; there are over 330 species alive today. Hummingbirds live in a wide range of habitats, and their diversity was likely fostered by the Andean uplift and the accompanying array of habitats freshly created along this altitudinal gradient. There is some suggestion that hummingbirds may have originated in lowland rainforest; this is where the center of diversity of a specialized group called the hermits is found (*79*). Hermits are cryptically colored and less diverse than other hummingbirds and were initially

thought to be the first group to branch off from the rest; however new findings now suggest that a larger, colorful group called the Topazes are perhaps the earliest hummingbirds to diverge (*80*).

European biologists, since their own emergence and diversification over the past few centuries, have long been intrigued by hummingbirds. Societies in the Americas were likely fascinated much earlier: among the famous Nazca lines in present-day Peru are hummingbird-shaped geoglyphs dating from 400-600AD —mysteriously akin to crop-circles, these are only fully comprehensible when viewed from the air (*81*). Treatises by German and British researchers on the hummingbird's long, fringed, and unexpectedly forked tongue were written as early as the seventeenth century (*82*, *83*). Hummingbirds, kept in captivity in zoos, puzzled German scientists in the eighteenth and nineteenth centuries who devised complicated models to explain the apparent straw-like suction that appeared to allow the birds to drink. Debate on the precise mechanism by which these tiny birds were able to drink nectar became heated in the early twentieth century before World War II, only to resurface almost a century later (*84*, *85*). Did they drink via capillarity? Gravity? Suction? Aided by high-speed cameras, recent research has only now revealed the intricate and complicated drinking mechanism. While capillarity may play a role in some situations, hummingbird drinking likely primarily involves passive nectar-trapping by the tongue's fringe-like lamellae (*86*, *87*).

This was not the first hummingbird surprise in recent years: high-speed videography has greatly enhanced scientists' ability to follow and to study even the most extreme aspects of the birds' secret lives. Some species produce low melodic sounds as they pull out of a U-shaped courtship dive--generated not with their vocal cords, but with the feathers of their tail (*88*). With hearts beating up to 20 times a second during exercise and wings that flap 200 times a second, hummingbirds are fast in everything they do—including feeding. High-frame-rate filming

revealed that previous estimates of tongue extensions of 2.6 times a second were gross underestimates (*89*). During feeding bouts, birds routinely extend their tongue at rates of up to 17 times a second (*90*). Try it—this is fast.

Given their extreme pace of life, it seemed likely that hummingbirds also possessed rapid metabolic capabilities. Biochemical work has shown that certain digestive enzymes in hummingbirds -- those involved in breaking down and transporting sugar -- have the highest rates of activity seen in vertebrates (*91*). This rapid capacity to assimilate sugars could have profound consequences, and might, theoretically, compensate for a missing taste. As hummingbirds readily come to feeders, they are amenable to experimentation, and biologists over the decades have performed many tests investigating their preference for various sugars and concentrations. Yet as it was never once suggested that hummingbirds might not taste sweet, all experiments were performed over periods of time in which rapid metabolic effects could be integrated: that sugar-high might kick in. However, writing in 1945, a biologist named Frank Bené described what most birdwatchers intuit: within 'a few quick sips' a hummingbird he watched rejected a solution with too low a concentration of sugar or honey (*92*), a reaction fast enough to likely be only a sensory response.

In this thesis, I present the results of research addressing this mystery. Chapter 1 details behavioral tests (Figure 4), Chapter 2 outlines molecular and bioinformatic studies, and includes the outcome of functional studies resulting from a wonderful collaboration. After years of trying to express the receptors in cell culture, I went to the International Symposium for Olfaction and Taste to look for help. I had the great fortune of meeting Professor Keiko Abe, who introduced me to Dr. Yasuka Toda, then a graduate student in the lab of Professor Takumi Misaka. Dr. Toda had pioneered a new cell-based luminescence assay and had success expressing the mammalian

umami receptor. We began a collaboration, and what we discovered through our joint efforts is that hummingbirds have a "re-purposed" savory receptor (*1*): many residues are changed in many parts of the receptor to allow it to be activated by a new class of chemicals. By tinkering with material remaining in the hummingbirds' tiny genome—the tiniest genome of any bird (*93*)—evolution fashioned an unlikely new vertebrate sweet receptor, and the hummingbirds' Umwelt broadened.

Why was the sweet receptor lost in the first place? Again, this is a matter for speculation from our modern, Neogene vantage point. However, two alternative just-so stories could be invoked. The first revolves around diet: mammalian meat eaters, such as lions and tigers and cheetahs, on the one hand, and, convergently, vampire bats, and other carnivores like the Asian small-clawed otter on the other, have also lost sweet sensation. There seems to be a link between a meat-centric diet and relaxation of selection pressure on the sweet-receptor—although in this instance, the causality might be reversed, as a single point mutation inducing a stop codon and preventing a receptor from being expressed might also have an effect on feeding patterns. As birds are the descendants of carnivorous theropod dinosaurs (*94*), the close relatives of T-Rex, similar connections between their dietary habits and their complement of taste receptors might explain the absence of *T1R2*. Alternatively, other neutral factors may have led to the gene's absence. Curiously, all flying vertebrates—birds, bats and extinct pterosaurs—show a similar pattern of genome-size reduction (*95*). In theropod dinosaurs, this occurred many millions of years before the evolution of birds, but there appears perhaps to be a link with the demands of flight: smaller genomes might mean smaller cells which could lead to more efficient oxygen transport, for instance. As bird gene families are on average smaller in size (*96*), a wholesale reduction could have also led to the loss of *T1R2*. Unlike in mammals, even a remnant version of

the gene (a pseudogene) isn't detectable in the bird genome, indicating that this loss happened a long time ago, early in the history of birds.

Once *T1R2* was lost, birds may have only been able to detect amino acids instead of sugars. However, bitter taste receptors are also present in bird genomes. In birds, this gene family is much smaller than in mammals, and avian bitter receptors are broadly tuned, meaning that these receptors can recognize a variety of different potentially toxic chemicals (*97*). Armed with these tastes, birds could use gustation similarly to mammals, to avoid toxic compounds and to detect and assess nutrients. This raises an interesting aside: is the hummingbird sensory vocabulary more limited than that of mammals — are savory and sweet combined into simply a single, delicious sensation, if the same receptor detects both? New evidence suggests this could be the case: Chapter 3 describes the studies Dr. Toda and I are embarking on to begin to address this question: this will hopefully lay the groundwork for future research examining how taste is processed in the avian brain, and how neural circuits evolve with dramatic shifts in diet and perception.

Many new questions are raised. Distantly related bird families are also sweet-sensitive: do these other birds taste sweet also with the savory receptor? Did the same base pairs mutate or did the tinkering modify different parts of the protein? And how did nectar-feeding evolve in the other groups? Some groups, like the Australian honeyeaters, are closely-related to birds like the insectivorous pardalotes that also feed on the carbohydrate-rich secretions of sap-sucking insects and the sugary exudates, called manna, of eucalyptus trees (*98*, *99*). While the routes and methods of sweet acquisition could be varied, the end results are the same: studying the mechanism of sweet perception is thus a thread that can be followed through the labyrinth of time, a window into the evolutionary process on the genetic level.

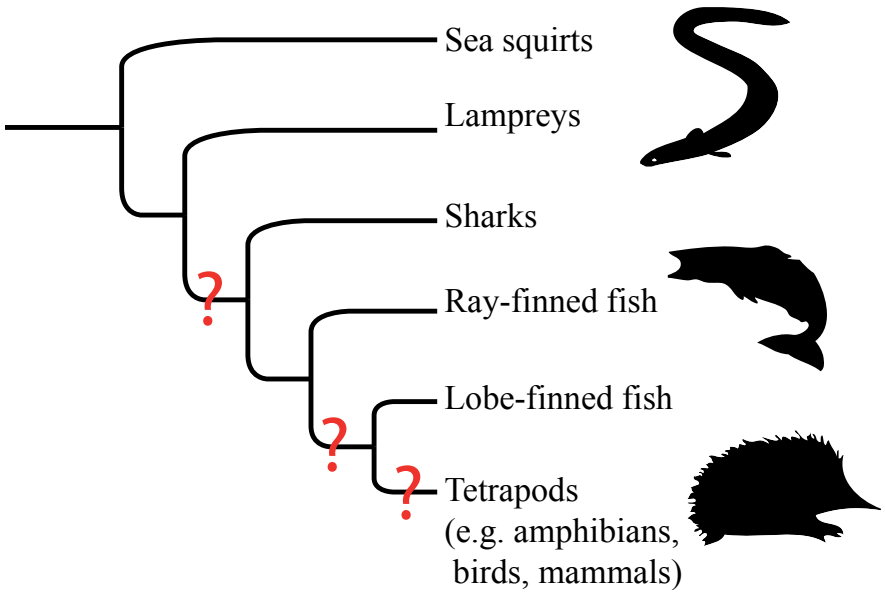


**Figure 5. Possible alternative origins of the mammalian sweet receptor.**

When did the mammalian sweet receptor evolve (Figure 5)? The taste receptors of some fish have been tested and do not seem to respond to sugars (*30*), so when the familiar, human sensation of sweetness first evolved is still unknown. Maybe lampreys and sharks could taste sugars and then some ray-finned fish lost this ability; perhaps sweet taste only first evolved after *Tiktaalik* left the water and four-legged tetrapods colonized the terrestrial part of our planet, by changes in the receptors of frogs or in the taste buds of the forefather of birds and mammals, the ancestral amniote. Or maybe true sweetness—as the artificial sweetener industry calls it, the "gold standard" of the sweet taste of sucrose—is only a mammalian construct, part of our hairy, toothed, endothermic Umwelt where we are cued in from an early age to sugars found in breast milk, exuding perhaps first from hair patches like those of monotreme echidnas, later transferred over the millennia via a variety of different ancestral nipple states into the mouths of our own human young.

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## Chapter 1

### Hummingbird detection of carbohydrates and sweeteners

Hummingbirds display a strong and immediate preference for many sugars that are sweet to humans. All ~330 species of hummingbirds are specialized nectar-feeders, and while their diet also contains high numbers of insects, hummingbirds' ability to sense carbohydrates has seemed self-evident to bird-watchers and biologists.

The lack of *T1R2* in the chicken genome raised the immediate question of how nectar-feeding birds perceive the dominant sugars in nectar—sucrose, glucose and fructose (*1-3*). Many lineages of birds are nectarivorous, but hummingbirds are both the most specialized as well as the most well-studied group: not only does nectar play a primary role in their diet, but an extensive literature exists on carbohydrate preferences (*3-7*), concentration preferences (*8*, *9*), and digestive enzymes (*10-13*). Moreover, hummingbirds represent an undisputed instance of the evolution of nectarivory from strict insectivory: hummingbirds are nested within the Strisores (*14*) and evolved from an ancestor which likely resembled their sister group, the entirely insectivorous swifts (*15-18*).

Hummingbird tongues have been the focus of much research (*19-25*). Yet little is known about hummingbird taste buds: a study on tongue musculature which sectioned portions of the tongue in several species revealed a single taste bud on the tongue base of an Allen's hummingbird (*Selasphorus sasin*) (*25*). The existence of bird taste buds, initially in question, was confirmed long after taste buds were described in other vertebrates (*26*, *27*). In most species, buds are small, fungiform-like and often difficult to distinguish histologically and are orders of magnitude fewer in number: chickens have approximately 300, primarily on the upper palate (*28*, *29*). Recently, the presence of a taste-specific g-protein, gustducin, has also been demonstrated in

a bird (*30*). Yet while the number and distribution of buds in hummingbirds is unknown, it is clear that in the bird species examined, the basic anatomical structures and signaling components necessary for oral taste sensing exist.

Yet after the possibility of a loss of *T1R2* was raised, it was necessary to re-evaluate whether hummingbirds were assessing sugar content with what Darwin called ‘‘real taste in the mouth” (*31*) or whether sweet perception in birds was fundamentally different than in mammals. One alternate possibility was that perception was perhaps mediated by a metabolic rather than a sensory response. Metabolic responses to caloric meals are well-documented and such compensatory feeding is common in birds: calorie-restricted jungle-fowl (the species from which domestic chickens arise), for instance, show a preference for a sucrose–supplemented diet (*32*). Many taste tests in birds were not designed as brief-access trials and monitored in real-time or video-recorded, as is the standard in studies of taste in rodents and other model systems, but were conducted over longer periods of time ranging from 15 minutes to over 48 hours.

Thus, while many behavioral studies describing hummingbird preferences (*3*, *7*, *8*) and drawing comparisons with other nectar-feeders (*11*, *33*, *34*) add to our understanding of the biology of these nectar-feeding-birds, many of these studies were not conducted on time-scales which allowed definitive exclusion of a dominant metabolic sugar-sensing mechanism rather than sensory perception. Taste has rarely been brought into the equation; it was likely assumed to operate as in mammals, if at all--some work refutes the importance of taste in hummingbird diet selection (*35*). A recent study showing thresholds for different carbohydrates in broad-billed hummingbirds (*Cynanthus latirostris*) elegantly demonstrated highly acute sensitivity as well as differences between gustatory thresholds for different sugars, yet as these trials were also longer in duration (consumption measured every 15 minutes) the possibility could not be completely

ruled out that the discriminating mechanism was not sensory (*7*). A study on black-chinned hummingbirds (*Archilochus alexandri*) in 1945 describes hummingbirds' ability to discriminate with 'a few quick sips' (*36*), yet systematic brief-access trials have not been conducted.

In addition to shortening the time-span of the stimulus presentation, another route of addressing whether the response to carbohydrates has a sensory component is by presenting stimuli that are sweet but have no caloric value. Artificial sweeteners, however, often fail to act across species, and even animals with a functional sweet receptor may not be able to detect a synthetic sweetener: most mice, for instance, are insensitive to aspartame (*37*) despite a functional T1R2/T1R3 heterodimer. This is not surprising: sweeteners are developed for human consumption, and changes in other species to other parts of the receptor not involved in activation by sucrose can alter the ability of the receptor to respond to the synthetic chemicals. For instance, early work showed that squirrel monkeys respond positively to dulcin but reject saccharin; for rats, the inverse is true (*38*). Work by Dieter Glaser shows variation in responses to a large panel of sweeteners by some insects and vertebrates (*39*).

A lack of a response to an artificial sweetener does not indicate a lack of a receptor to detect sugars, but a positive, appetitive response is strong evidence that the animal detects a sweetener via a sensory rather than a metabolic mechanism. Sweeteners are not neutral and may have metabolic effects as well (*40*, *41*)—these are also thought to act via T1R taste receptors expressed in the gut. However, only a few studies have examined the role of sweeteners in birds—one such study demonstrates that ravens reject saccharin (*42*). In hummingbirds, only one report has been published, to our knowledge: Stromberg and Johnson tested preferences of black-chinned hummingbirds (*Archilochus alexandri*) to aspartame and saccharin and did not see a positive response (*43*).

Thus, from the literature, it was unclear if in any bird, preference for a 'sweet' compound could be definitely uncoupled from a metabolic response to caloric content. In a single potential exception, Bernard Weischer, (in a paper published in German, in 1965) examined taste preferences in birds and lizards—one of the only other tests of non-carbohydrate sweeteners in birds. He showed an aversive response in parrots towards saccharin and dulcin, but a preference for a sugar alcohol called mannitol (*44*). Sugar alcohols, also called polyols, are hydrogenated carbohydrates, many of which occur naturally in fruits. Many are 'partially-nutritive'—mannitol is described as a "low-calorie" sweeteners with some metabolizable energy (*45*), and to humans, it is reported to be about 25% as sweet as sucrose on a molar basis (*39*): even here, support for a sensory response is equivocal.

Another complexity surrounding sweeteners is that they can activate other taste receptors as well: in the case of acesulfame K and saccharin, bitter receptors are activated in addition to the sweet receptors (*46*, *47*). This could be one of the factors explaining why many artificial sweeteners are described by humans as having a bitter or an 'off-taste' (*48*). The sweet receptor of rats is activated in cell culture by sucralose (*49*), yet unless sucralose is presented with a maltodextrin stabilizer (as in the commercial product Splenda ®), sucralose is rejected by many individuals in behavioral trials (*50-53*). How a single compound can be both 'bitter' and 'sweet' may at first seem counter-intuitive but as recent work (*54-56*) in *Drosophila* shows, bitter taste often can suppress sweet perception (*57*, *58*).

Using behavioral data to understand how sweet perception could be detected by an unknown sweet receptor is, therefore, quite complicated. A lack of a response does not indicate a lack of a general sweet receptor, and could either be due to an inability of the sweetener to activate the receptor, or to the action of the sweetener on additional receptors mediating aversion.

A positive response to a non-caloric stimulus is relatively straightforward, but the evidence for multiple, allosteric binding sites of sweeteners also complicates the interpretation of which domain or subunit is involved. Concentration is also a key factor: as bitterness ratings increase with concentration (*48*), finding the appropriate concentration is critical.

In addition, in testing scenarios when multiple stimuli are presented simultaneously, the relative concentrations are also important. Hummingbirds will sample feeders and will drink longest from the highest concentration of sucrose, another possible confounding factor if the sweetener is presented simultaneously with a sucrose feeder as a control. Relative sweetness values determined from human psychophysical tests (*39*) can serve as an initial reference but there is no guarantee that the perception of other species does not differ in this regard.

Nevertheless, despite potential confounds, a positive response to a sweetener would present evidence of a sensory-mediated detection of sugar, so in initial field experiments at the Lytle field station in Utah, we tested a panel of sugars and sweeteners. These experiments on black-chinned hummingbirds presented here (Part 1), informed later tests on the congeneric ruby-throated hummingbirds (*Archilochus colubris*) in Massachusetts (Part 2). Part 2 also describes high-speed trials designed to determine discrimination speed, also performed on captive individuals of this species. Part 3 describes tests with artificial sweeteners on a mixed population of another "bee" hummingbird, Anna's hummingbirds (*Calypte anna*), together with black-chinned hummingbirds and Allen's hummingbirds, in California. As experimental design, location, and species varied, they are presented here separately. Part 2 and 3 include experiments included in our publication (*59*). Part 3 also includes additional trials with Splenda ® not discussed in this paper.

## Part 1: Taste preferences of black-chinned hummingbirds (*Archilochus alexandri*) in Utah

### Methods

Hummingbird bioassays were performed on a wild population of black-chinned hummingbirds at the Lytle ranch (Santa Clara, UT, USA) following a previously-published experimental design used to assess taste preferences (*60*). Six disposable feeders containing 10 ml of fluid each were placed in a circular array and observed from a distance of $> 3$ meters; most trials consisted of three stimuli presented in duplicate. Trials lasted 1 hour, and approximately every 15 minutes, the array was rotated to prevent the formation of spatial memories and the development of position preferences. Trials were observed and behavior scored in real-time by an observer equipped with binoculars and blind to the identity of the composition of the feeder. For each stimulus presented, bout length was recorded to the nearest second.

Proportion of long bouts (>1 second) (Figure 6A) and bout mean (Figure 6B and Figure 7, mean ± SE) were recorded for each trial. Most trials consisted of simultaneous presentation of three solutions: the stimulus alone, the stimulus mixed with sucrose, and sucrose alone. Different classes of potential sweeteners were tested, including amino acids (Figure 7A), synthetic sweeteners (Figure 7B), sugar alcohols (Figure 7C) and sucrose of different concentrations (Figure 7D). In most instances, sweeteners were presented at concentrations roughly equi-sweet to 500 mM sucrose to humans (*39*). Aversion was determined by a reduction in bout mean in the mixture of the stimulus and sucrose, when compared to the sucrose control. Differences in bout mean between the mixture and the sucrose control were assessed using Student's *t* tests (Figure 7A-C) or one-way ANOVA to compare bout mean between concentrations (Figure 7D) (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

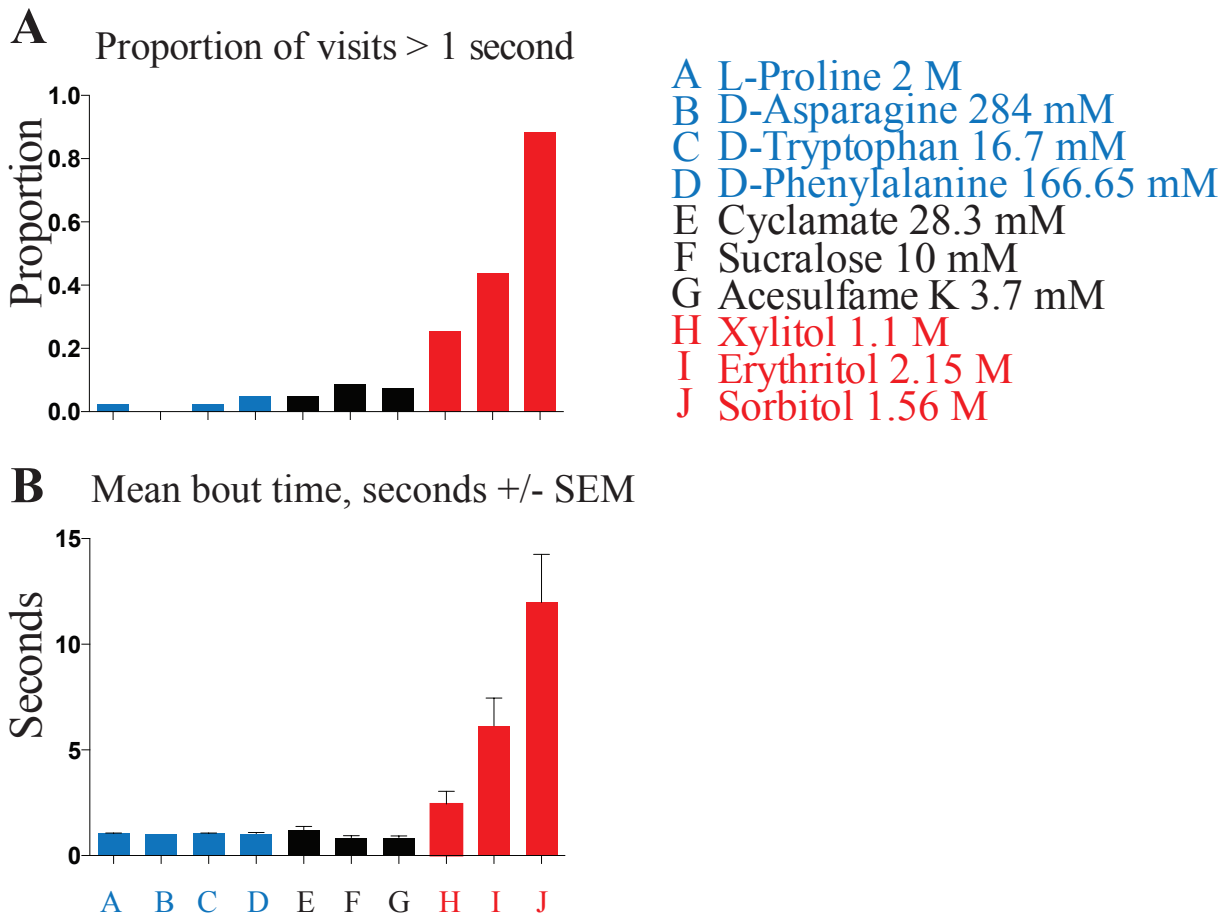


**Figure 6. Sugar alcohols elicit preference behavior in black-chinned hummingbirds.**

**(A):** Summary of experiments testing preferences of a wild population of black-chinned hummingbirds (*Archilochus alexandri*) presented with a panel of potential sweet compounds. Amino acids are shown in blue, synthetic sweetners in black, and sugar alcohols in red. The ratio of long bouts to total number of bouts for sugar alcohols (red bars) was higher than for any other compound. **(B):** Summary figure showing mean bout length (± SE) for a panel of sweeteners. Samples sizes are listed in the Appendix.

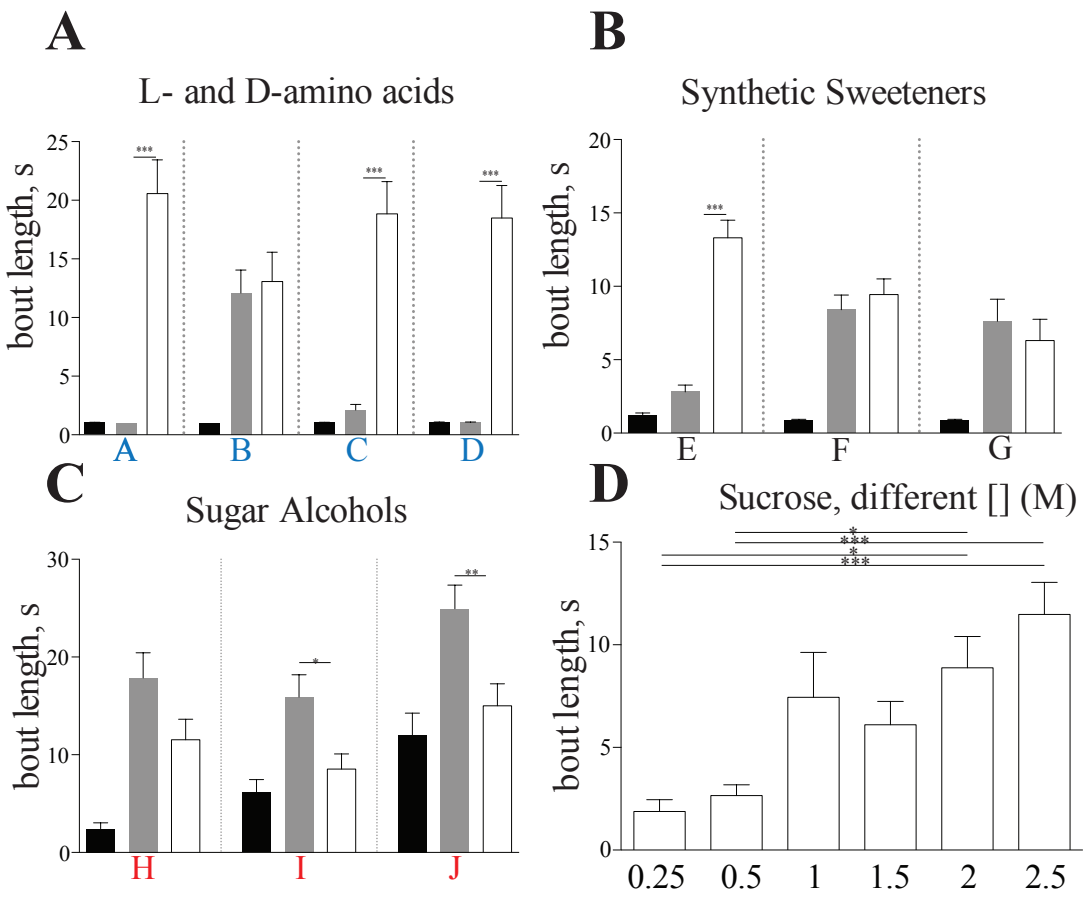


**Figure 7. Black-chinned hummingbirds exhibit varying responses to different classes of chemicals. (A-C):** Different classes of chemicals (synthetic human sweeteners, L and D-amino acids, and sugar alcohols, labeled A-J as in Figure 6) were presented either alone (black bar) or in combination with 500 mM sucrose (grey bar) simultaneously with feeders containing 500 mM sucrose (white bar) as a control, and bout time was recorded. A reduction in bout time between the sucrose control and the mixture indicates an aversive response; (mean ± SE, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; significance assessed with Student's *t* test, samples sizes in Appendix). Concentrations were chosen to present stimuli at approximately equi-sweet concentrations to 500 mM sucrose**. (D)**: Simultaneous presentation of varying concentrations of sucrose (M) (mean ± SE, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; one-way ANOVA, samples sizes in Appendix).

**Results and Discussion**

The results of these experiments show that at the concentrations we tested, most sweeteners were either neutral or aversive: with the exception of the sugar alcohols erythritol and sorbitol (Figure 6). Sorbitol, like mannitol used by Weischer, is partially nutritive, but erythritol is marketed as 'zero-calorie' and is not known to be metabolizable (even by bacteria, hence its inclusion in 'natural' toothpastes as a sweetener). Thus, unless hummingbirds were able to metabolize erythritol, this suggested a sensory rather than metabolic response.

Some stimuli were aversive at the concentrations tested, as indicated by a decrease in consumption when presented together with sucrose. However, the context of the presentation matters: when presented simultaneously (Figure 7D) the solution containing the highest concentration of sucrose elicited the longest bouts. Thus, a sweetener might be 'sweet' or appetetive, but if it is less potent than 500 mM sucrose, the preference might not be detectable. However, simultaneous presentation with sucrose was necessary both to maintain the motivation of the birds to participate and to consistently sample all feeders, as well to control for variation in overall feeding rates due to extrinsic factors (timing, weather, etc.).

As these experiments were pilot trials, a number of experiments had to be discarded due to technical problems (such as tests with aspartame and neotame, not shown here). Sucralose was inadvertently tested at 10 mM rather than 100 mM (less than equisweet to 500 mM sucrose). Additional attempts to test preferences for other carbohydrates were discontinued due changing population sizes of birds and insufficient visit numbers. These experiments were also recorded by *in situ* observations: unlike trials in Part 2 and 3, no video was taken. Potential variations in individual behavior such as an apparent preference for cyclamate displayed by one individual (either a female or juvenile male) remain exciting but anecdotal. In a pilot test with a sample of

Envision ®, a specialty product designed and donated by Domino Sugar, the hummingbirds' response did not appear to be inhibited by lactisole, a sweet taste inhibitor that acts on human T1R3. Hummingbirds also did not show a preference for a commercial preparation of Stevia (*61*), which was aversive, as were the concentrations of cyclamate and the amino acids (except D-asparagine) tested here.

**Part 2: Experiments with captive hummingbirds: brief-access tests and high-speed trials**

Together with swifts, hummingbirds formerly formed an order called the Apodiformes (*62*). As their name suggests, they have tiny legs and feet; thus, regulations restrict color-banding in the US. In our wild behavioral assays, therefore, we cannot distinguish individuals and are merely able to record the sex and species of a visitor. To examine the preference behavior of individual birds in a setting in which we could track individuals and monitor previous consumption behavior, we tested the taste preferences of 4 ruby-throated hummingbirds (*Archilochis colubris)*, a species closely-related to black-chinned hummingbirds, and the most common species on the East Coast of the United States. Wild-caught birds were captured and housed for flight studies at Harvard's Concord Field Station (CFS) in Bedford, Mass and were maintained in flight cages. Over a period of a week in June 2009, I performed a series of repeated taste trials. Here, only two primary stimuli were used: the putative sensory receptor agonist, erythritol, was used, as well as aspartame, which had been reported to elicit no response in wild black-chinned hummingbirds (*43*).

**Methods**

For brief-access behavioral trials, the birds (n = 3-4) were tested simultaneously with 2 ml cuvettes filled with 333 mM sucrose solution and with a test stimulus: 2.15 M erythritol, 3

mM aspartame, (both approximately as sweet as 500 mM sucrose to humans) or water. A slightly lower concentration of sucrose was used to make it less attractive than the sweetener, so that a preference would not be masked by the presence of a sweeter alternative. To maintain motivation to feed, water was not used as the paired stimulus: birds could potentially exhibit reduced motivation to sample if neither cuvette had a preferred solution for too many consecutive trials. 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. Paired stimuli were presented in 2 ml cuvettes filmed at 30 frames per second (FPS) for approximately 5 minutes after the first drink was taken. Each bird received multiple (2-8) brief-access trials of each stimulus, and the side the stimulus was presented on was alternated between trials.

Three parameters were extracted from the recordings: 1) bout length 2) number of long bouts (> 1 second) per minute, and 3) percentage of time spent drinking (Figure 8). The presence of long bouts is a better indicator of preference than bout mean, because birds briefly sampled both feeders (increasing the variance) but only stayed for long drinks at the feeder with a preferred stimulus. Drinking bouts were scored as the time between drinking onset, defined as when the bill tip entered the feeder, and the initiation of withdrawal behavior.

For high-speed trials to measure discrimination speed (Figure 9A), 5 birds were presented simultaneously with water and a sucrose solution (500 mM), and their behavior was filmed with a Photron 1280 camera at 500 frames per second. The number of licks and the time until water was rejected was recorded as mean per bird over 2-3 trials (Figure 9B and C).

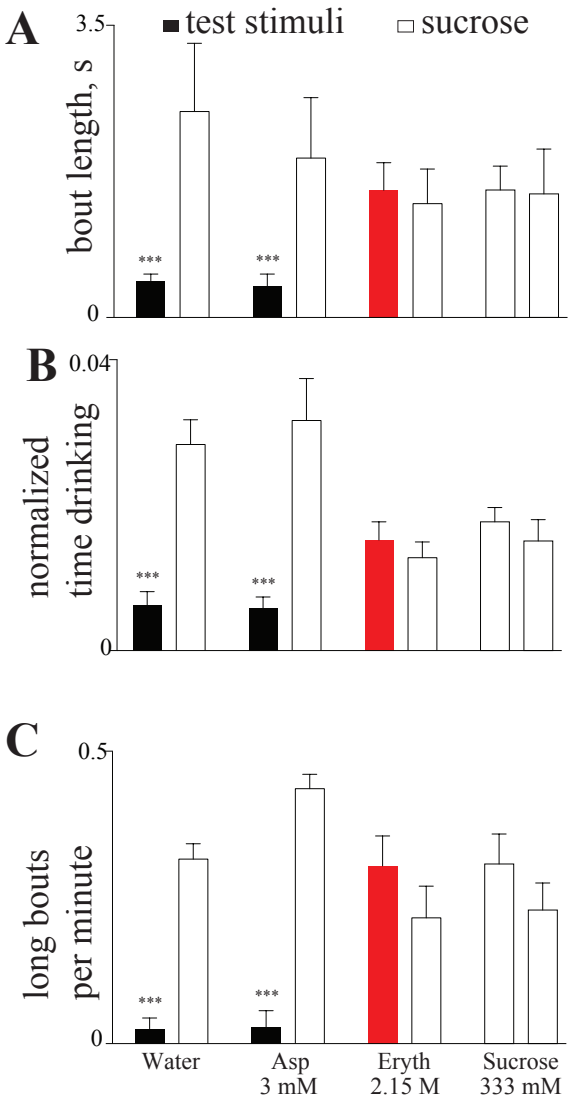


**Figure 8. Responses of captive ruby-throated hummingbirds (*Archilochus colubris*).** Captive ruby-throated hummingbirds (n = 3 - 4, mean ± SE) were presented with feeders containing test solutions (aspartame, erythritol or water) and sucrose (333 mM), and the drinking bout length **(A)**, time drinking **(B)**, and number of long bouts( (>1 second) recorded **(C)**, (linear mixed-effect models for differences between stimuli/sucrose, ***p≤0.001). Red bars indicate feeding bouts similar to those elicited by carbohydrates. When sucrose was present in both feeders (rightmost pair of bars) no difference in these parameters was observed.

**Results and Discussion**

In ruby-throated hummingbirds, erythritol elicited behavioral responses similar to those elicited by the sucrose solution: the average bout length, the total time spent drinking, and the number of long bouts all were similar to responses to the paired sucrose control. Water and aspartame were not preferred; birds merely sampled the feeders briefly. Figure 8 shows the results of these captive trials. In this figure, each parameter is averaged across birds, with a value for each bird determined on a per trial basis. 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 ($\alpha=0.05$).

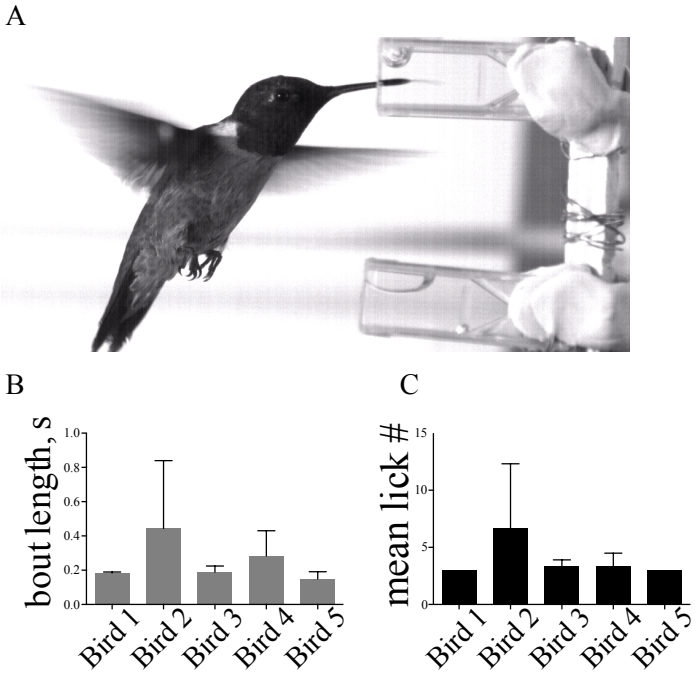


**Figure 9. Ruby-throated hummingbirds discriminate rapidly between water and sucrose.** **(A)**: An image from a high-speed video of a ruby-throated hummingbird rejecting water presented in the top cuvette after three tongue licks (162 milliseconds). Sucrose (500 mM, bottom cuvette) elicits a prolonged feeding bout. **(B, C):** On average (mean +/- SD, 5 birds, 2-3 trials per bird) rejections occurred within ~250 ms, or 3 - 4 tongue licks.

With two statistics consultants (Steven Worthington and Simo Goshev) at the Institute for Quantitative Social Science (IQSS) at Harvard, we designed a generalized linear mixed model to account for multiple testing of individuals and to control for order effects. We obtained similar results to the analysis with the linear mixed-effects model: the amount of time drinking erythritol (compared to the total drinking time per trial) is significantly larger than the amount of time birds drank water (compared to the total drinking per trial); (erythritol-water coefficient estimate = 1.11, std. error 0.1217, $p<0.001$***, Tukey contrasts for multiple comparisons of means).

**Figure 10. Rejection of an aversive stimulus by a rapid, characteristic behavioral response.** A ruby-throated hummingbird in a high-speed trial takes 3 licks of a sucrose solution containing a commercially-available food-coloring, and exhibits a rapid, characteristic rejection response involving headshaking and spitting. This response was similar to responses seen in lower frame-rate filming of taste trials in the field.

For high-speed trials, the mean rejection of water occurred within 250 milliseconds. Most birds rejected water immediately, switching feeders after 3 licks or ~160 ms, but in some trials, birds paused with their bill in the liquid before withdrawing, increasing the overall mean. Mean bout time before rejection, by bird, is shown in Figure 9B.

Together, these results in Part 2 show that the response to erythritol exists in another species and in an experimental set-up where individual identity is monitored. No preference for aspartame at 3 mM was observed. High-speed tests indicate that the detection of sucrose occurs very rapidly, suggesting a sensory rather than a metabolic response.

Other high-speed trials were performed in 2011 as part of a separate collaboration on hummingbird tongue shape and nectar uptake with Francois Peaudecerf (*15*). During these pilot trials, we tested responses of a small number of birds to 2 mM quinine and saw in some instances marked aversion. The behavioral pattern of head shaking and spitting was also observed to food coloring, added to the feeders to visualize the rise of the fluid in the tongue grooves. The rejection response may be mediated by bitter taste receptors or by other detectors of chemical irritants. The trigeminal response (chemisthesis) differs in terms of specific ligands that are noxious to birds compared to mammals (*19*) but this sense is nevertheless functional in birds (*21*). Anecdotal rejection after tasting food coloring is shown by still images from the high-speed video in Figure 10. We observed similar headshaking and rejection behavior in wild populations of Anna's hummingbirds (*Calypte anna*) (*59*) to certain artificial sweeteners, in trials where birds were filmed at a lower frame rates (60 frames/second) (Part 3).

Bitter taste receptors in chickens, turkeys and zebra finches have recently been functionally characterized and appear to be broadly tuned (*63*). A search of the newly released Anna's hummingbird genome reveals the presence of multiple putatively functional bitter

receptors. Because hummingbirds appear sensitive to aversive components in nectar it will be interesting to examine the response profiles of T2Rs in Anna's hummingbirds, as well as to examine the potential role of trigeminally-mediated rejection behavior.

## Part 3: Behavioral tests of wild Anna's hummingbirds (*Calypte anna*)

### Methods

Because our subsequent molecular work (described in Chapter 2) used taste receptors cloned from a third species, Anna's hummingbirds (*Calypte anna*), we decided to test the preferences of this species as well as to investigate the extent to which T1R1/T1R3 agonists cause behavioral attraction in wild birds. In addition, we discovered that another sweetener, sucralose, which was tested previously only at a lower concentration in the field, activated the receptors in cell-culture: thus, we wanted to assess the response of hummingbirds to higher concentrations of sucralose. Experiments were conducted in the Santa Monica Mountains in Topanga, California, USA, on the property of UCLA professor Thomas Smith and Brenda Larison, who graciously allowed me to occupy their porch and test the birds visiting their feeders. Most individuals were Anna's hummingbirds or black-chinned hummingbirds, but occasional visits by Allen's hummingbirds (*Selasphorus sasin*) were also recorded.

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 that did not touch the solutions and could be reused. Stimulus presentation was designed so that birds were required to feed while hovering, and so that the feeder was the appropriate depth and width for solutions to still be reached by the birds' tongues after 15 minutes of feeding. Feeders for experiments are shown in Figure 11A and

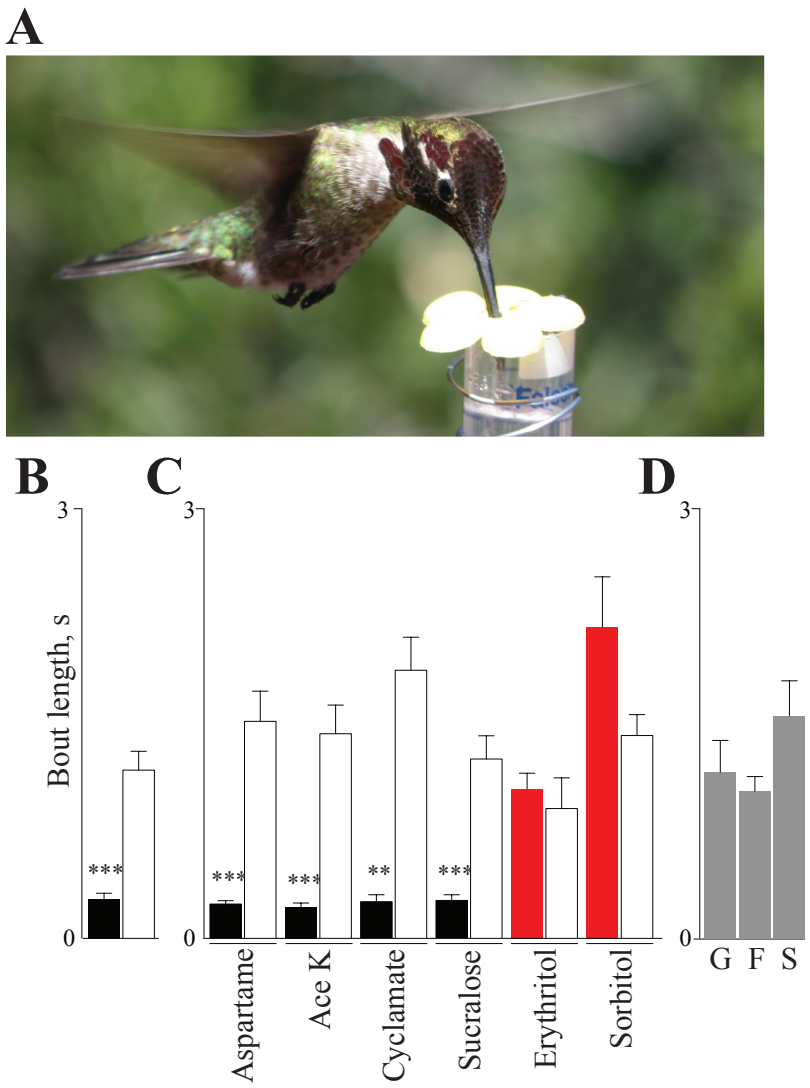


**Figure 11. Taste preferences of wild Anna's hummingbirds for T1R1-T1R3 agonists.** The taste preferences of wild Anna's hummingbirds **(A)** were measured (mean bout lengths ± SE), (Kolmogorov-Smirnov tests to compare mixtures and sucrose solutions: *p≤0.05, **p≤0.01, ***p≤0.001, sample sizes in Appendix). Sucrose (500 mM, white bars) was presented simultaneously with test stimuli (black); which were either **(B)** water or **(C)** a test compound (aspartame, 15 mM; acesulfame K, 50 mM; cyclamate, 30 mM; sucralose, 10 mM; erythritol, 2.15 M; sorbitol, 1.56 M). In **(D)**, 1 M carbohydrates (glucose (G), fructose (F) sucrose (S)) were tested simultaneously. Red bars indicate equal preference between sucrose and stimuli.

contained either water, human sweeteners, or carbohydrates (concentrations listed in Figures 11-13). 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. Multiple individuals of the three species of hummingbirds 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 in the trials performed in April (2013) and June (2014). 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 (Figure 11), sucrose (Figure 11), and the mixture (Figures 12 and 13) and were scored from the videos as the time between entry of the bill tip into the feeder and the initiation of withdrawal behavior. 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).

In these experiments, the concentration of the stimulus is extremely important, because, even for an agonist, presentation of the stimulus at too low a concentration would render the sweetener imperceptible, and too high concentrations are also aversive. To ensure that we tested the appropriate range of concentrations, for the sweeteners for which no preference was observed, we tested both low and at high concentrations. As in the trials in Utah, aversion was measured by a decrease in drinking from a mixture of the sweetener with a sucrose control. 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 (Figure 12 and 13). In Figure 12, mean bout length for Anna's males as well as for the whole population of birds is shown for non-preferred sweeteners at concentrations ranging from neutral to aversive. Experiments involving sucralose (1 mM)

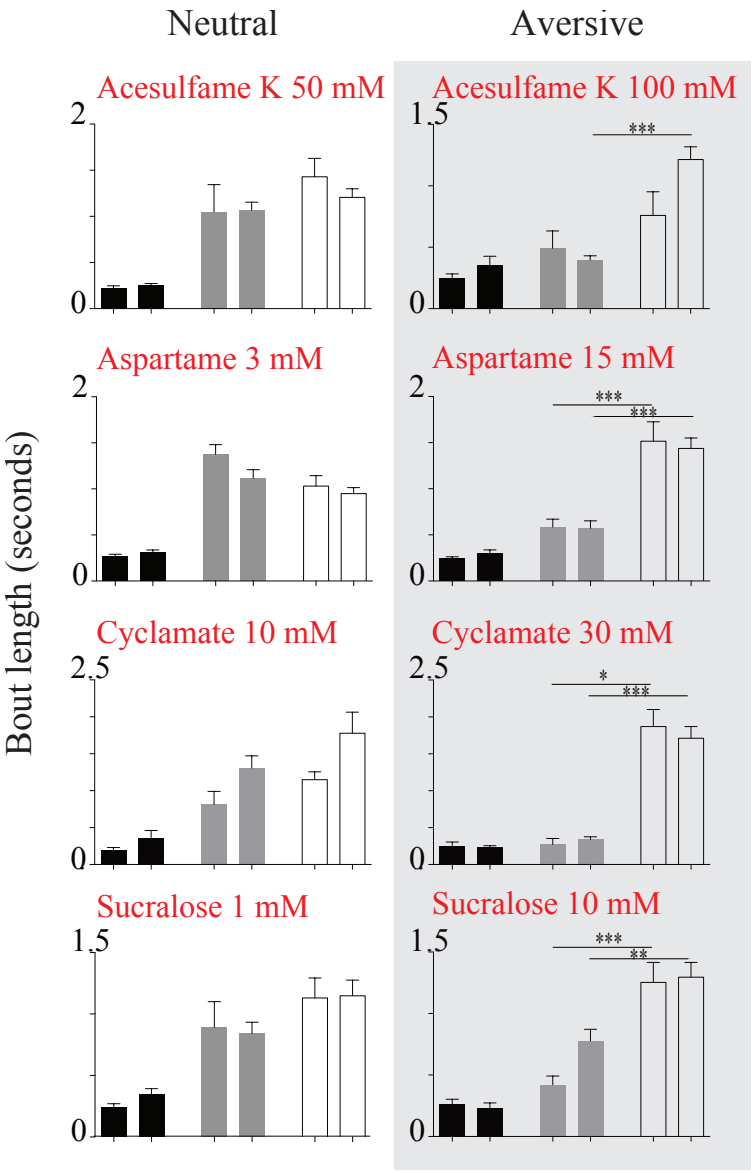


**Figure 12. Synthetic sweeteners for humans are aversive to birds at high concentrations.** Response of wild hummingbirds to human sweeteners at low and high concentrations. Bout lengths of male Anna's hummingbirds (left bar) and all hummingbirds (right bar) to stimuli (black bars), a mixture of stimuli and 500 mM sucrose (grey bars), and a sucrose control (white bars) were determined (mean ± SE, Kolmogorov-Smirnov tests to compare mixtures and sucrose solutions: *p≤0.05, **p≤0.01, ***p≤0.001, sample sizes in Appendix).

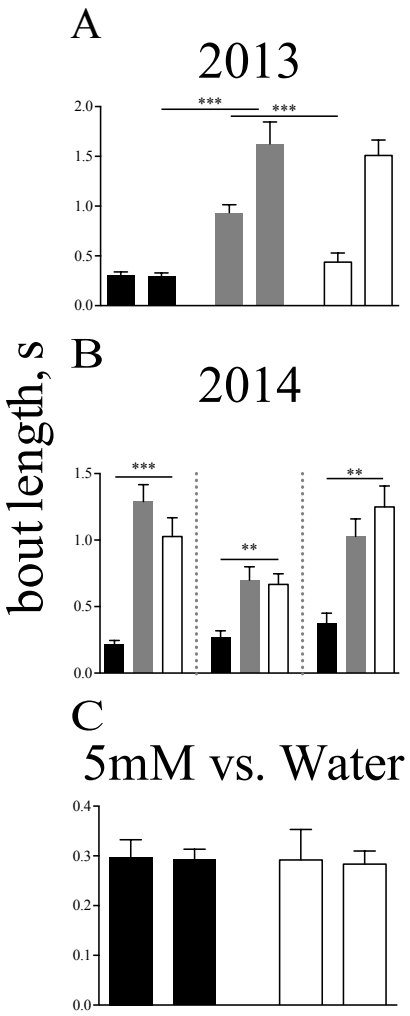


**Figure 13. Responses of Anna's hummingbirds to 5 mM sucralose in different field seasons and experimental designs.** Bout lengths of male Anna's hummingbirds (left bar) and all hummingbirds (right bar) to 5 mM sucralose (black bars), sucrose (500 mM, white bars), and a mixture of 5 mM sucralose and 500 mM sucrose (grey bars) were determined (mean ± SE, Kolmogorov-Smirnov tests to compare mixtures and 5 mM sucralose to the sucrose control: **p≤0.01, ***p≤0.001). **(A)** Result obtained in 2013 indicating a possible preference for the mixture in a subset of the birds. **(B)** Three separate trials showing slight variation in 2014, but no preference. **(C)** 5 mM sucralose presented only with water (Kolmogorov-Smirnov tests to compare 5 mM sucralose to the water control, no difference).

were tested in a subsequent field season (2014). One bird with a deformed bill and aberrant drinking behavior was removed from the analysis of Figure 12, sucralose, 1 mM, and from Figure 13.

In a subsequent field visit (June 2014), the response to sucralose at lower concentrations was assessed in more depth. Initial trials with low concentrations of sucralose had yielded confusing results which possibly suggested a preference among the Anna's males for the mix of sucrose and sucralose at 5 mM (Figure 13A). We re-tested these concentrations (Figure 13B) and also tested 5mM sucralose presented together only with water (Figure 13C) to see if any detectable preference existed in the absence of sucrose.

We also tested Splenda ® (Figure 14), because results from rats showed rejection of sucralose (despite receptor activation) but a preference of Splenda ® (sucralose plus a stabilizer). Two different experimental set-ups were used to test Splenda ® preference: one experiment was performed in which the equivalent of 0.55 mM and 1.38 mM sucralose solutions were presented simultaneously, and in a second experiment, the Splenda ® equivalent of 2.22 mM sucralose was paired only with water.

**Results and Discussion**

In large part, T1R1/T1R3 agonists were preferred by the birds, providing evidence for the role of this modified ancestral umami receptor (Chapter 2) in guiding hummingbird taste behavior. Mean bout length is presented in Figure 11 for male Anna's hummingbirds: sugar alcohols as well as carbohydrates were appetitive, and synthetic sweeteners that did not activate the receptor in cell culture were not preferred at any concentration tested (Figure 12). High concentrations of all synthetic sweeteners were aversive.

The one discordant result was that of sucralose: in cell culture, it activated the receptor pair, yet to the wild birds it appeared to be aversive. The data from 2013 (Figure 13A),

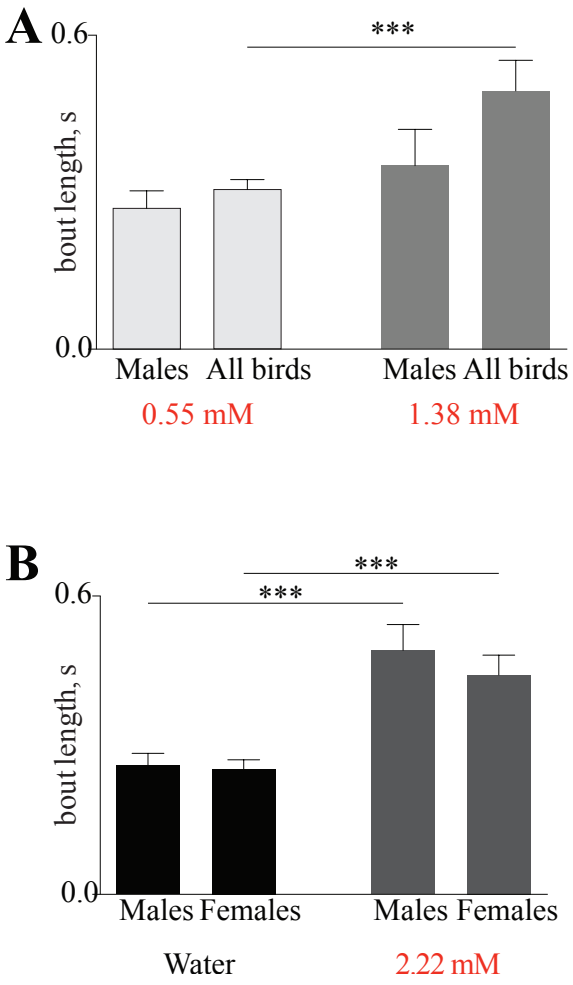


**Figure 14. Splenda ® is preferred by Anna's hummingbirds. (A)**: Higher concentrations of Splenda ® (equivalent to 1.38 mM sucralose) elicited longer drinking bouts than trials with lower Splenda concentrations (equivalent to 0.55 mM sucralose) in the combined population of birds, when presented without sucrose or water feeders. **(B)**: Bout lengths of wild hummingbirds at feeders containing Splenda ® at a concentration equivalent to 2.22 mM sucralose were higher than bout lengths at feeders with water (mean ± SE, Kolmogorov-Smirnov tests: ***p≤0.001m, sample sizes in the Appendix).

however, raised the following question: did 5 mM sucralose, when paired with sucrose, somehow represent a 'sweet spot'—at this concentration, were bitter effects masked by sucrose?

In experiments from 2014, however, (Figure 13B) we did not find evidence for a preference (or an aversion) of the mix compared to the sucrose control among Anna's males or in the larger population. When presented only with water as a paired stimulus, sucralose was not preferred, indicating that it wasn't a matter of a weak preference being masked by a preference for the 500 mM sucrose feeder. Together, these results suggest that when presented at this concentration, it is either below threshold or perhaps still aversive. Thus, we found no evidence for any preference of sucralose, despite activation of the T1R1/T1R3 receptor by sucralose in cell culture.

In rats, interestingly, a similar rejection of sucralose has also been observed in some individuals—despite the fact that sucralose is a T1R agonist in heterologous systems. When researchers gave rats Splenda ®, the commercial version of sucralose containing a "stabilizer" of either maltodextrin or dextrose, rats that had formerly rejected sucralose preferred Splenda ® solutions; the stabilizer somehow removes the aversive side-effects, although the mechanism is not yet known. The molecular mechanism of the aversion to sucralose has not yet been described, but it might be due to sucralose acting on a bitter receptor. Other sweeteners activate bitter receptors—for mammals, this has been clearly shown for saccharin, as well as for acesulfame K (*46*), a sweetener that the birds in our experiments also rejected.

To begin to investigate whether Splenda ® had similar effects in hummingbirds as in rats, we tested different concentrations of Splenda ®. Splenda ® is marketed as sucralose mixed with maltodextrin and also sometimes with dextrose (glucose), depending on the formulation. A recent study using HPLC (*64*) determined granular Splenda ® to be composed of 1.1% sucralose,

1.08% glucose, 4.23% moisture, and 93.59% maltodextrin. Splenda ® 'packets', which we used, contain as a stabilizer either maltodextrin alone or maltodextrin and dextrose.

We did not observe rejection behavior at the lowest concentrations of Splenda ®. When two concentrations were simultaneously presented (0.55 mM and 1.38 mM) (Figure 14A) a slight preference for the higher concentration was observed among the cohort of all visiting birds. A clear preference for a higher concentration of Splenda ® (2.22 mM sucralose) when paired with water was exhibited by both sexes (Figure 14B).

The sweetness (or caloric value) of the stabilizers could be responsible for the response we observed. Alternatively, this could also represent an activation of the heterodimer by the sucralose while the buffering agent somehow partially masks the bitter response. In future work, we hope to investigate the functional response of T2Rs in Anna's hummingbirds as well as probe the behavioral and heterologous systems with higher concentrations of Splenda ® and the stabilizing agents, to understand the contribution of the maltodextrin component and to begin to get a better explanation of the puzzling aversive response to sucralose.

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## Chapter 2

### Taste receptor cloning, expression and bioinformatic analysis

Parts adapted from *Evolution of sweet taste perception in hummingbirds by transformation of the ancestral umami receptor* Science 22 August 2014: 345 (6199), 929-933. [DOI:10.1126/science.1255097]

Which receptors do hummingbirds use to sense sugars? After our behavioral experiments indicated that sensory receptors, rather than merely digestive enzymes, were likely involved, we decided to search for genes expressed in the oral cavity. Yet T1R2 is required for the only known vertebrate sweet receptor: if this gene was missing, there were no obvious alternative candidate genes.

Several small lines of evidence, however, suggested that the other *T1R* gene family members might be interesting candidate genes to investigate functionally. First, one study of T1Rs in cell culture proposed that T1R3 might act as a carbohydrate sensor at very high concentrations (*1*), raising the possibility that T1R3 could act as a homodimer. Biochemical assays as well as data on artificial sweeteners also suggested a role of T1R3 in sweetener sensing: in a binding assay, extracellular domains of both T1R2 and, surprisingly, T1R3 receptors showed affinity to carbohydrates and sucralose (*2*). In addition, data regarding the role of T1R3 in the detection of artificial sweeteners and the binding of lactisole (a sweet taste inhibitor) indicate that T1Rs have multiple interaction sites and neither subunit is functionally inert (*3*, *4*). Key residues for activation occur in all regions of both proteins: aspartame, monellin, and neotame interact with the extracellular (venus flytrap) domain of T1R2 (*3*, *5*). In T1R3, the neoculin response depends on residues in the extracellular domain (*6*), the response to brazzein requires sites in the cysteine rich region of T1R3 (as well as the venus flytrap domain of T1R2) (*4*, *7*), and the cyclamate and lactisole response depends on residues in the transmembrane

domain of T1R3 (*3*, *4*). Key residues can be sites where ligand is bound, or might be amino acids involved in inter-subunit interactions (*2*, *5*).

A final piece of evidence supporting a putative role of other T1Rs in bird sweet perception was from functional work in fish. In zebrafish and medaka, T1R2 and T1R3 together respond to amino acids, as do T1R1/T1R3 (*8*). It has been suggested that the ancestral T1R heterodimers were amino acid receptors (*8*, *9*) and that the ability of T1Rs to respond to carbohydrates in mammals evolved later. It is equally possible that the responses seen in zebrafish represent a derived, teleost state and that lobe-finned fishes and possibly sharks are sensitive to sugars. Many Family C GPCRs (the family of GPCRs that *T1R*s belong to, including pheromone receptors, calcium receptors, and neurotransmitter receptors (*10*) are amino acid receptors, including the only member of the family for which a crystal structure has been solved, the metabotropic glutamate receptor, mGluR (*11*), which binds glutamate. Reconstructions of the primordial Family C receptor indicate that this ancestral receptor was an amino acid receptor as well (*12*). Since the shift from an amino-acid response to a carbohydrate response occurred once before, it seemed possible that it could recur. Even if bird T1Rs were not carbohydrate sensors, additional studies characterizing the amino-acid response profile of T1R1/T1R3 would be of interest, as the only available functional data for the *T1R* gene family were from studies on primates, rodents, and fish. Thus, at minimum, we hoped to characterize the umami receptor response in a bird.

**Cloning of avian *T1Rs***

We obtained domestic chicken samples (single comb white leghorns) from the UC Davis breeding flock. Oral tissue samples were collected from Anna's hummingbirds (*Calypte anna*)

from wildlife rehabilitators in the Sacramento area (Wildlife Care). The third species that we cloned the *T1R*s from was a chimney swift (*Chaetura pelagica*) which we collected from Cambridge, MA.

From palate and tongue tissue, we generated total RNA (with Qiagen's RNEasy kit for fibrous tissue). *T1R*s were amplified from first-strand cDNA (Smartscribe, Clontech) (Figure 15). Avian *T1R*s were initially challenging to amplify, due perhaps to the low number of taste buds in birds, as well as the keratinized nature of much of the avian oral epithelium, and perhaps most importantly, the GC-rich nature of the 5' end of these receptors, especially *T1R1*. As the chicken genome was the only bird genome available at the start of the project, we used methods like Rapid Amplification of cDNA Ends (RACE) (*13*) or genome-walking to amplify the genes with universal primers tagging the ends of cDNA transcripts or fragmented genomic DNA.

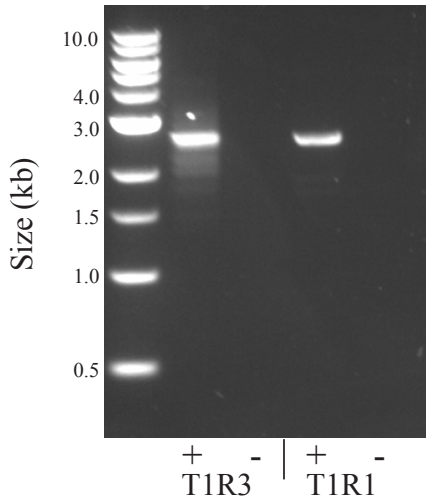
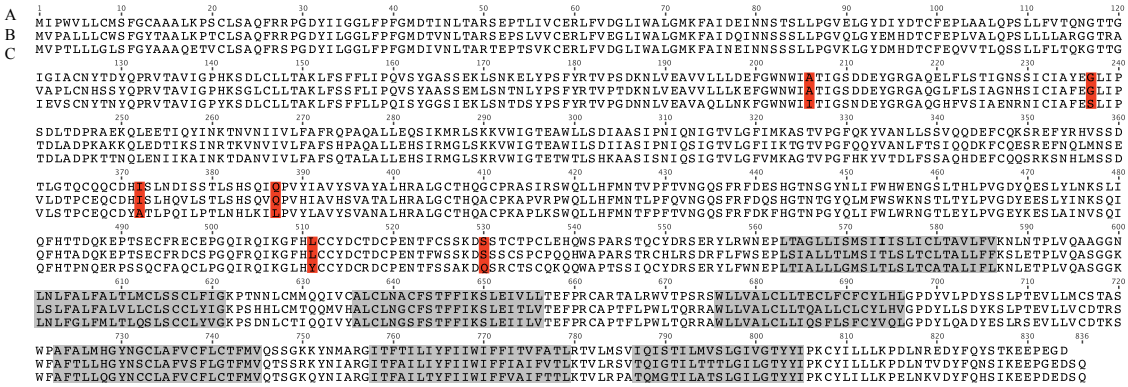



**Figure 15. Expression of *T1Rs* from Anna's hummingbird oral tissue.** Amplification of full-length, spliced *T1R1* and *T1R3* coding sequence from hummingbird oral tissue cDNA. cDNA reactions were prepared with (+) or without (-) reverse transcriptase.

T1R3



T1R1

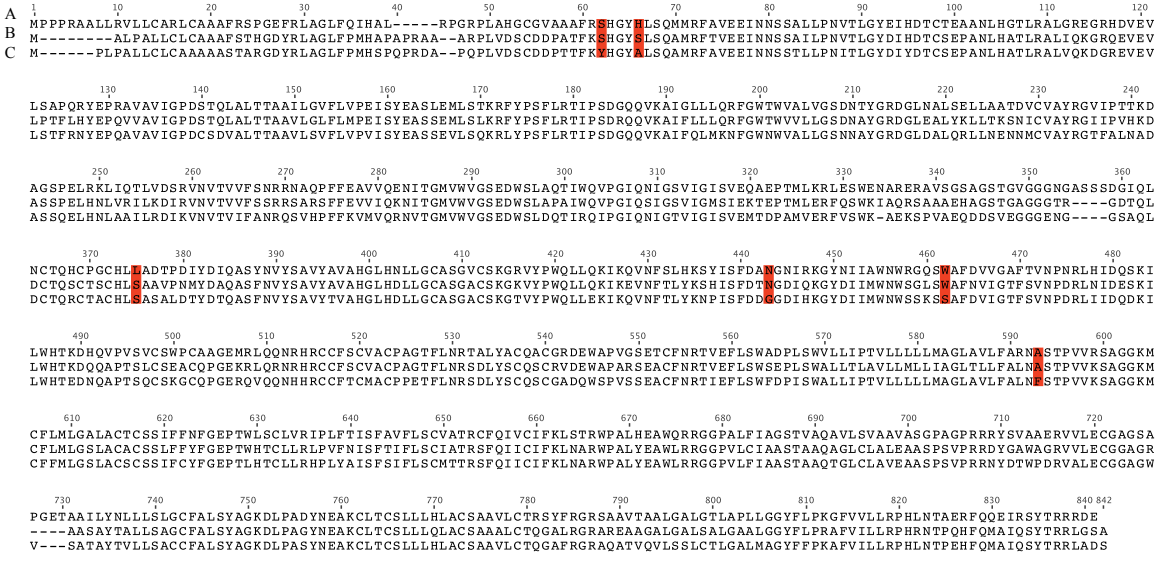


**Figure 16. Sequences of avian *T1R3* and *TIR1.***

Chicken (A), and swift (B) and hummingbird (C) *T1R3* and *T1R1* genes were aligned. Grey bars show putative transmembrane domains; red highlighting indicates candidate sites of positive selection (posterior probability > 0.5), as described later.

For PCR amplification, we used a polymerase designed for GC-rich sequences (Advantage ® GC 2 Polymerase) from Clontech, with primers with high (>69°C) annealing temperatures. The full-length, translated sequences for these three birds are shown in Figure 16.

**Functional expression of avian T1Rs**

Once we obtained the full-length sequence of *T1R1* and *T1R3*, our main objective was to test the function of this heterodimer. In collaboration with Dr. Yasuka Toda in Professor Takumi Misaka's lab at the University of Tokyo, we assayed the responses of these genes in heterologous expression assays using a novel luminescence method Dr. Toda and Professor Misaka had designed for mammalian T1Rs (*14*, *15*). Human 293T cells were transfected with plasmids containing a jellyfish derived photoprotein (mt-apoclytin-II), mouse Gα15, and pEAK10 mammalian expression vectors containing bird *T1R* sequences. 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, followed by the Holm adjustment for multiple comparisons ($\alpha=0.05$).

We tested responses of T1R1/T1R3 heterodimers to a panel of ligands (Figures 17 and 18): chicken and swift T1Rs responded strongly to amino acids, and like the mouse amino acid receptors, they did not respond to carbohydrates at any concentration (Figure 18A). Hummingbird receptors, by contrast, were strongly activated by increasing concentrations of carbohydrates, as well as by sucralose (Figure 17 and Figure 18A), and were weakly activated by amino acids and sugar alcohols. This receptor pair not did respond to other synthetic sweeteners such as cyclamate, aspartame, or acesulfame-K, or to L-proline (which activates mouse T1R2/T1R3).

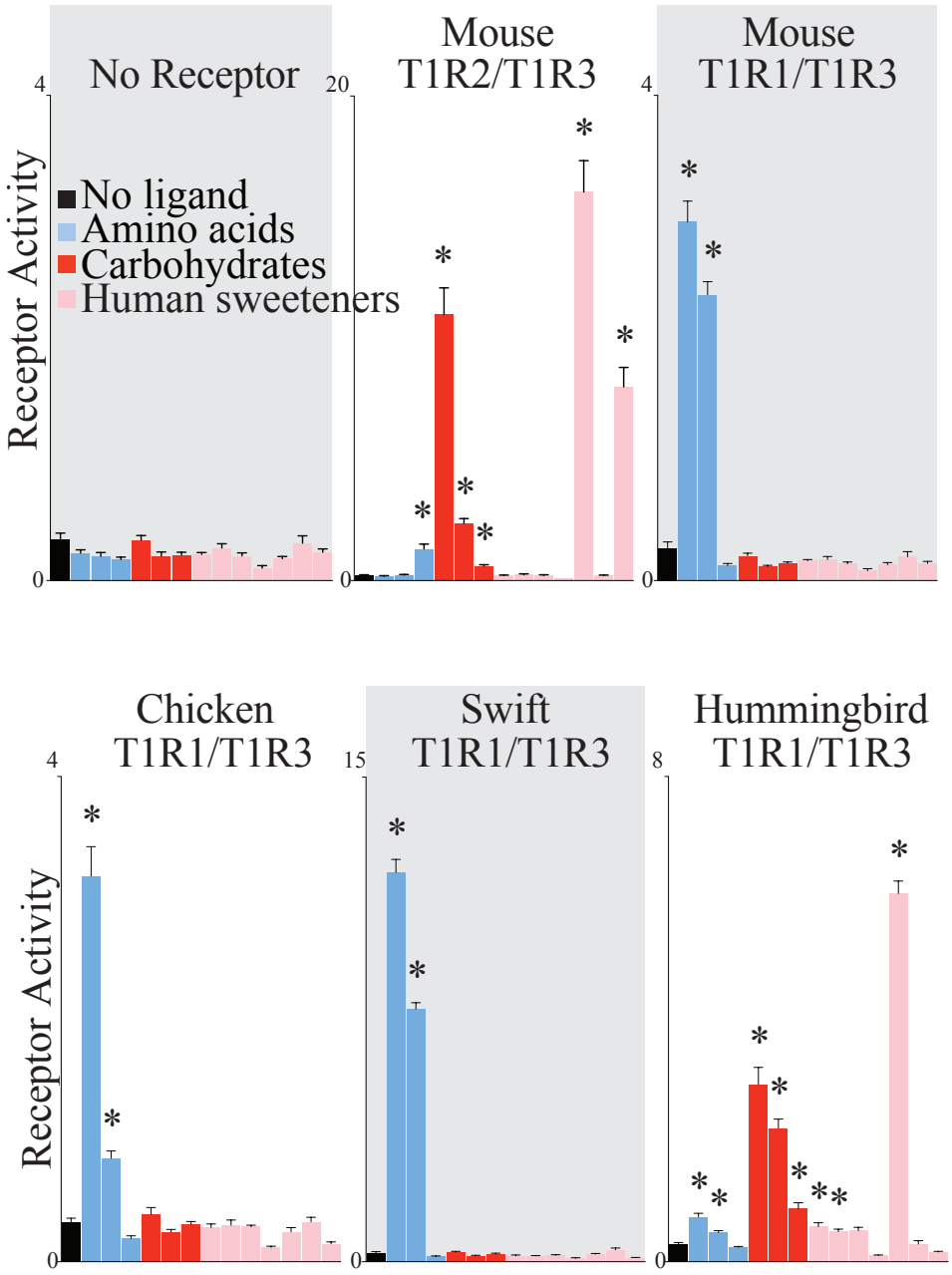



**Figure 17. Functional responses of T1Rs.** Functional expression of avian and rodent taste receptors to stimuli (in order: no ligand, L-alanine, L-serine, L-proline, sucrose, fructose, glucose, sorbitol, erythritol, xylitol, aspartame, sucralose, cyclamate, and acesulfame K; all 100 mM, except aspartame: 15 mM, n=6, *p≤0.05).

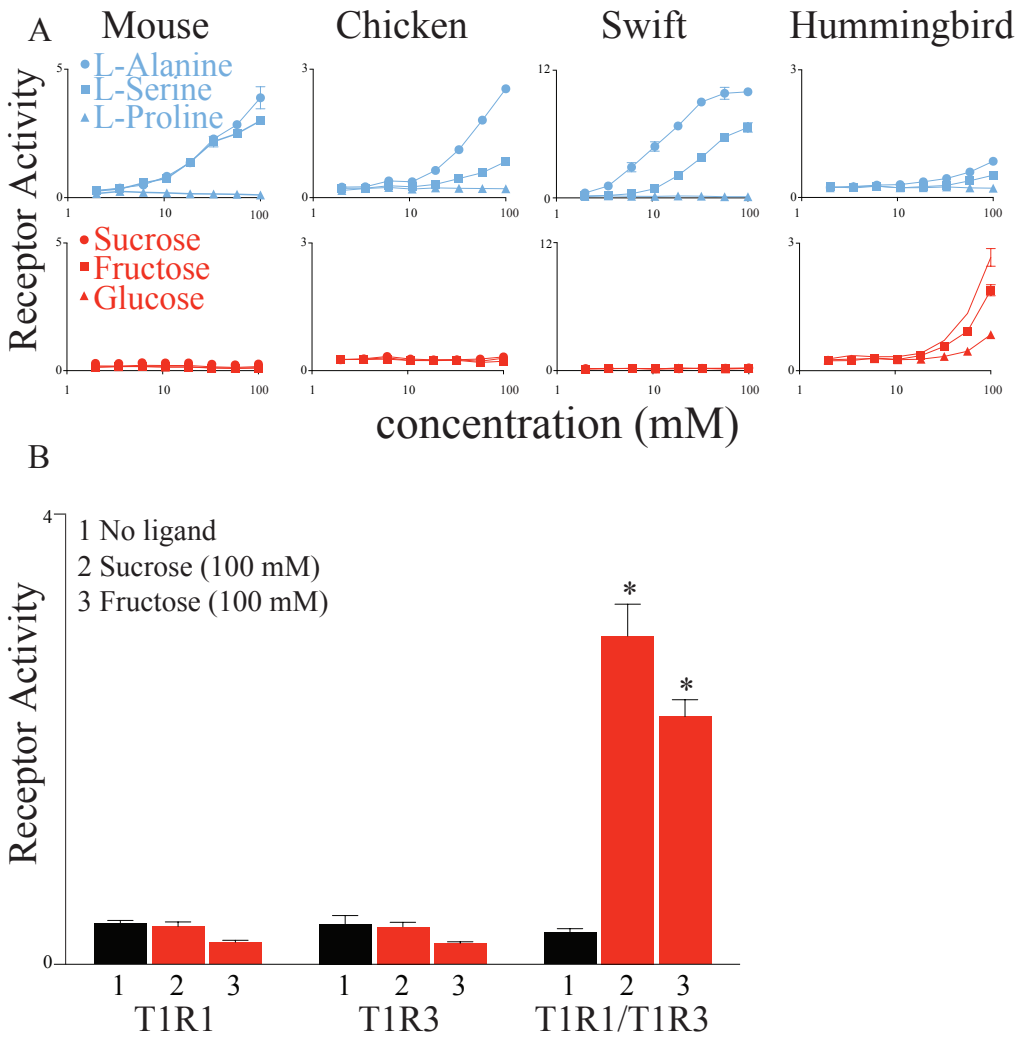



**Figure 18. Responses of the T1R1-T1R3 heterodimer. (A)** Dose-dependent responses of T1R1-T1R3 from species indicated to amino acids (blue) and sugars (red). **(B)** Responses of hummingbird T1Rs alone or in combination (n=6, *p≤0.05).

Contrary to our initial prediction, T1R3 did not act as a homodimer: T1R1 was necessary for the response to sugars (Figure 18B). In addition, hummingbird T1R1 was also specifically required: experiments using chicken T1R1 showed a strong response to L- alanine and a greatly reduced response to sucrose (Figure 19), indicating that both subunits were involved—either for ligand binding or for appropriate coupling between the heterodimers or g-protein activation.

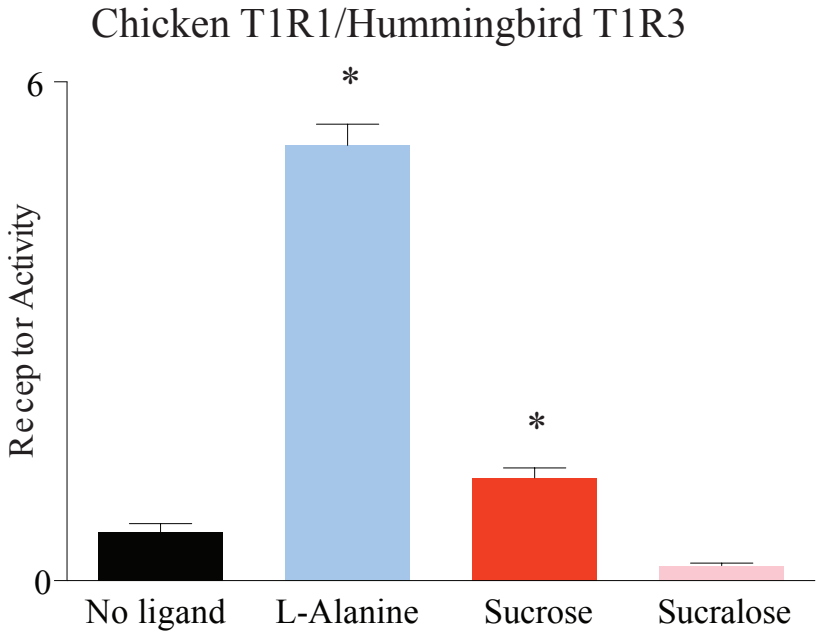



**Figure 19. Hummingbird T1R1 contributes to sugar responsiveness**. Cells expressing chicken T1R1 and hummingbird T1R3 were analyzed for responses to L-alanine, sucrose, and sucralose (each 100 mM) using the cell-based assay (n=6, mean ± SE, *p≤0.05, Welch's *t*-test). Hummingbird T1R1 is also required for maximal responsiveness to sugar.

To decipher which residues were involved in this drastic functional change, we generated a series of chimeras composed of chicken and hummingbird sequences (at this point in time, we had not been able to obtain tissue or *T1R* sequences from swifts). We discovered that replacing the 5' venus flytrap region from the hummingbird *T1R3* with the chicken sequence abolished the response to sugars, indicating that crucial residues existed in this domain. Further dissection of this extra-cellular region revealed a smaller subset of 109 residues containing key sites (Figure 20A and B). Within this region, 19 non-consecutive amino acids were finally identified, clustering in three areas on the protein surface. When the 19 sites were reintroduced to the venus flytrap domain of the chicken, sensitivity to sucrose and to sucralose was restored (Figure 20C).

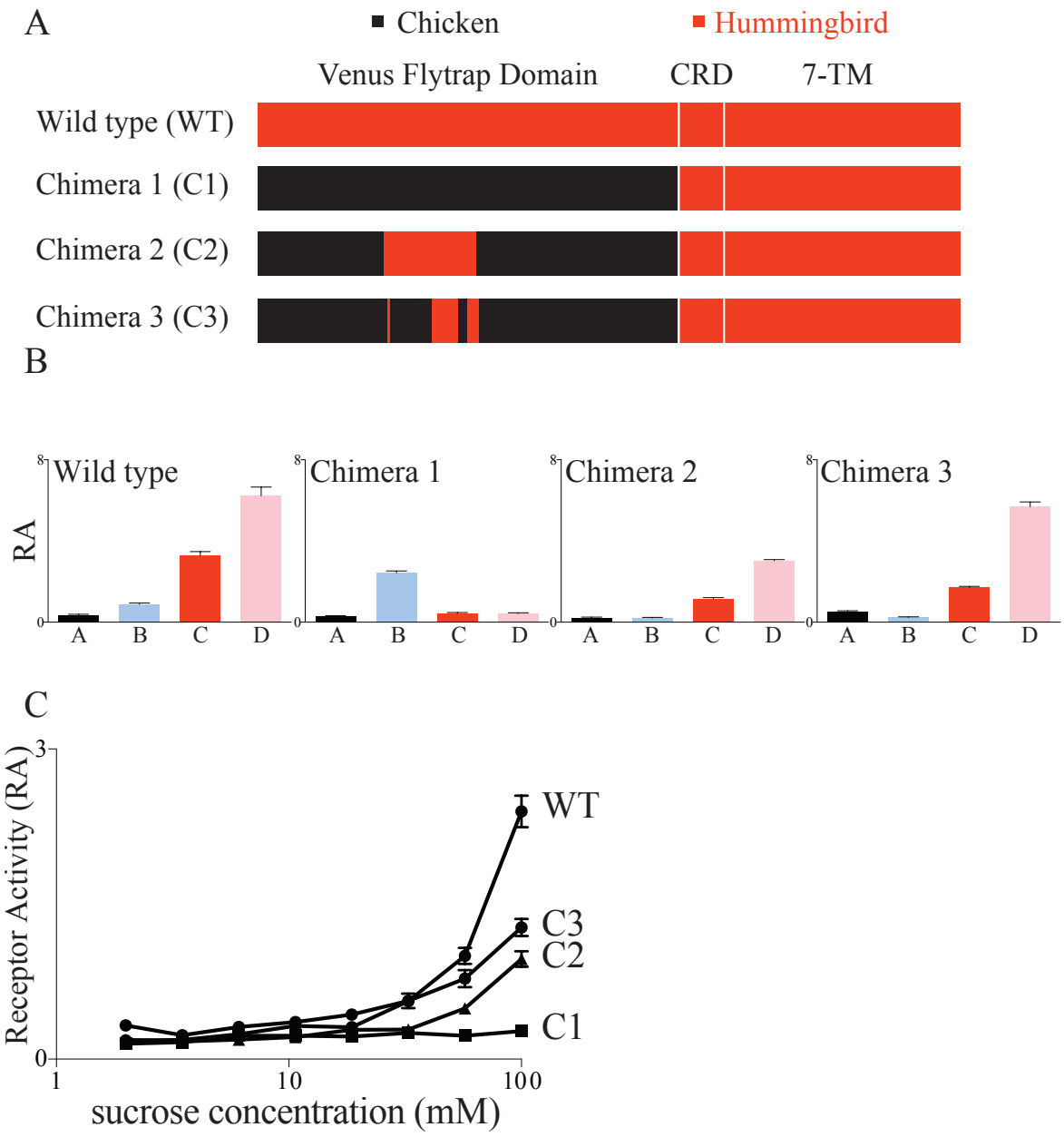



**Figure 20. Molecular basis for the responsiveness to sugars in hummingbird T1R1-T1R3.** **(A)** T1R3 chimeras containing chicken (black) and hummingbird (red) amino acids were designed (CRD: Cysteine-rich domain; TM: transmembrane domains). **(B)** Responses of T1R3 chimeras and hummingbird T1R1 to A: no ligand; B: 100 mM L-alanine; C: 100 mM sucrose; and D: 100 mM sucralose. **(C)** Dose-dependent responses of T1R3 chimeras and hummingbird T1R1 to sucrose.

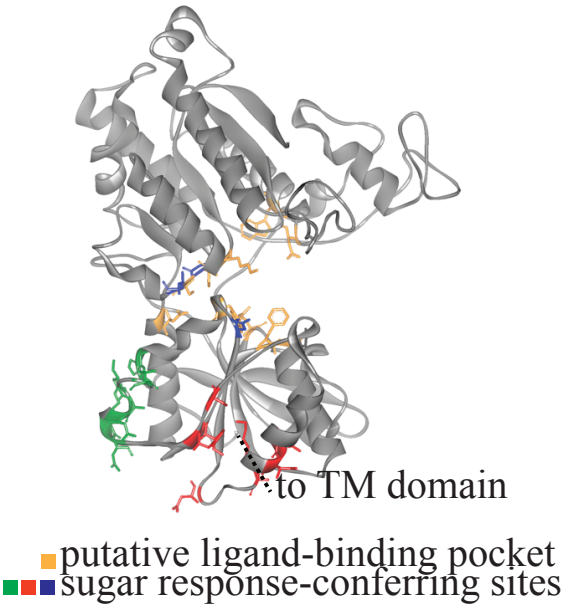



**Figure 21. A homology model of the venus flytrap domain of T1R3**.

The mutations that confer sugar responsiveness cluster in three distinct locations (red, green, blue). The putative ligand-binding site is shown (yellow), predicted by alignment with ligand-contacting sites of rat mGluR1.

Tomoya Nakagita of the Misaka lab constructed a homology model (Figure 21) to visualize these residues. The 19 sites cluster in three distinct regions: one small group by the putative ligand-binding pocket, and in two other clusters on the lower lobe, which are perhaps involved in interactions between T1R1 and T1R3.

In the comparison with swift sequences, 14 of these sites differ between hummingbirds and swifts. 12 sites are unique in hummingbirds, and 4 of these sites are different between all three birds. Investigating the roles of subsets of these mutations in the background of a reconstructed ancestral receptor (see below) will provide fascinating avenues for future research

and may shed light on basic questions about the evolutionary process such as the steps by which a complex novel function could evolve.

**Bioinformatic analyses: verification of *T1R2* loss**

In the bird genomes that were initially sequenced by the international community (chicken, zebra finch and turkey), *T1R2* was not identified by other authors (*16-18*), despite the presence of this gene in mammals, anoles, and in fish, where it is sometimes duplicated (*19-22*). A key aspect of the claim that *T1R2* was missing in birds, however, was the verification that this region was adequately covered in the focal genome. To confirm loss of *T1R2*, the presence of genes that flank *T1R2* in mammals (*Pax7* and *Aldh4a1* from Chromosome 4 in mouse) were documented to be present as well as syntenic in chickens (on Chromosome 21) (*16*) (Figure 22, from (*23*)).

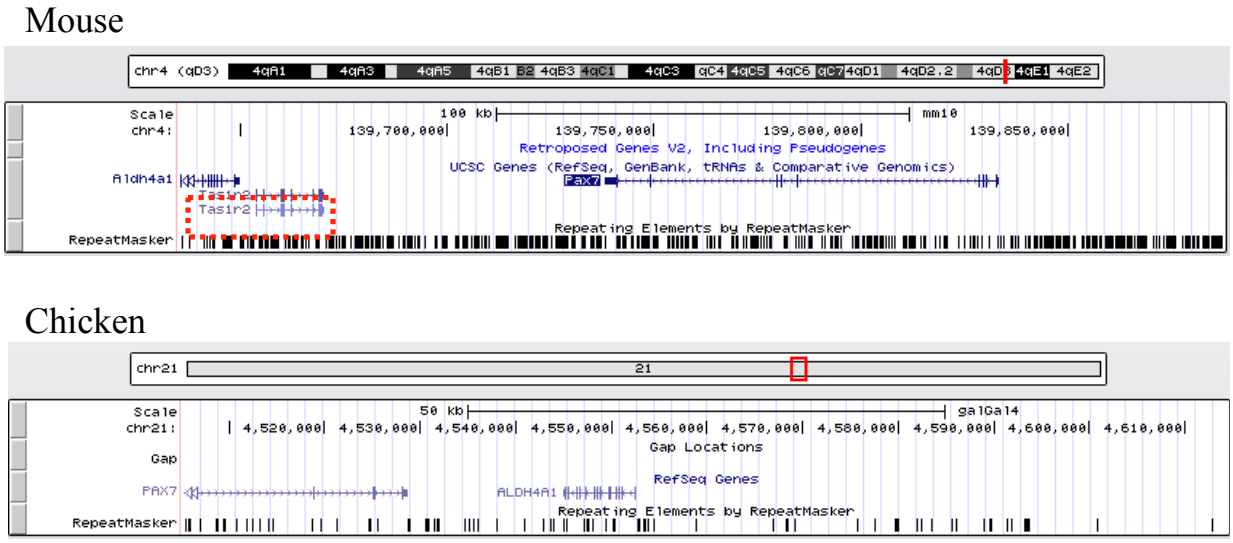



**Figure 22. Presence and synteny of flanking genes (*Aldh4a1* and *Pax7*) surrounding *T1R2* in mice and in chickens.** *T1R2* is found on Chromosome 4 in mice; in chickens, these two genes are Chromosome 21. In the intervening ~16 kb of sequence we found no remnant of *T1R2*. Image from UCSC Genome Browser.

In the current release of the chicken genome (Galgal4) no gaps exist between the flanking loci, however in early versions of the chicken genome, small gaps had not yet been filled between these genes. We ordered the corresponding bacterial artificial chromosome (BAC) as well as the BAC that spanned *T1R1* from the Children's Hospital Oakland Research Institute (CHORI). As part of a larger sequencing run, we obtained Illumina reads from these clones. Full coverage was not obtained but the sequences tiled the region, and none of the assembled contigs contained sequence that did not map to the area: there was no evidence of a missassembly or a undiscovered gap in this region. In addition, no contig contained anything that aligned to *T1R2*. The region between the flanking loci is approximately16,000 bases, and we verified that no genes are predicted to fall in this region using online tools such as Genscan (*24*). In addition, while we also Illumina-sequenced the BAC spanning the first exons of *T1R1*, the resulting contigs did not cover this region: sequencing through these gaps and further upstream of the first exon of *T1R1* are goals for the future.

**Widespread absence of *T1R2***

Gene loss is more difficult to prove than gene gain because a gene might exist but the locus may simply be challenging to amplify or sequence. Initial experiments with Southern blotting to verify the loss of *T1R2* did not prove straightforward; finding a probe to reliably amplify a single copy across species but not hybridize to putative paralogs was difficult. We decided instead to take a bioinformatic approach; in 2013, additional bird genomes began to be published so we analyzed 10 additional bird species, widely distributed across the avian tree, as well as the genome of the Chinese alligator.

We searched 11 archosaur (bird and alligator) genomes for the presence of *T1R2* using human *T1R2* as queries. In local TBLASTX searches (version 2.2.26+) (*25*) we used an e-value cut-off of 0.01 and verified hits by reciprocal BLAST to Genbank (NCBI) (*26*). In all birds, the best hit from searches for *T1R2* reciprocally blasted to other Class C GCPRS. In the majority of cases, the top hits were to contigs containing the *T1R1* or *T1R3* sequence we had previously extracted. In a small number of cases the *T1R*s spanned multiple contigs, in which case we downloaded the contigs and aligned them, individually, to the exons for human *T1R2* using the software Geneious (R7, Biomatters), then extracted the aligning regions and blasted them to the nr-database (NCBI). This analysis identified these hits as other class C GPCRS (*GPR6A*, *CASR*, and metabotropic glutamate receptors): and in no case did we detect a genuine *T1R2* sequence in any bird genome tested. In the alligator genome, the best hit was clearly *T1R2*, verifying its presence in this group and providing evidence that a mammalian *T1R2* is an appropriate query to identify *T1R2* in an archosaur, if it were present in the sequenced genome.

To ensure that these results were not due to missing sequence at this region, we also used the flanking locus approach (*16*, *27*) and performed a reciprocal BLAST analysis of the genes known to flank *T1R2* in mammals (*Pax7* and *Aldh4a1*) against the 11 downloaded genomes, with sequences downloaded from the chicken genome as queries. In all archosaur genomes in our dataset we were able to verify the presence of these genes, although contig size was often too small to assess synteny.

**Signatures of selection in *T1R1* and *T1R3*: assembling *T1R*s from genomes**

To assess whether there was evidence of positive selection in the hummingbird *T1Rs*, we assembled a dataset of 68 predicted *T1R* coding sequences by analysis of online databases or by experimental protocols described above for hummingbirds, swifts, and chickens. Online sequences were obtained either from Ensembl (*28*), Genbank (*26*), or where no prediction was available, by local BLAST analysis on 11 downloaded genomes, as described below. Our preference was to extract Ensembl sequences, but if none were present for a particular species, then the Genbank prediction was used. If multiple transcripts were predicted, the one that best aligned and that contained the highest coverage over the whole alignment was chosen (i.e. the longest transcript with the least truncation on either end). Minor modifications not relevant to our analysis were made to the predicted sequences, including removing terminal stop-codons and sequence upstream of the start codon.

Since we cloned the *T1R1* and *T1R3* genes for chicken, hummingbird and swift, we had reliable and high quality sequences for these species. However for all other archosaurs, the online predictions were often less reliable and contained gaps or fragments of other genes, rendering it necessary in many instances to extract these sequences manually. As described for the analysis of *T1R2* absence, genomes were downloaded from NCBI and local TBLASTX searches were performed, this time using each individual exon from our cloned hummingbird sequence for *T1R1* and *T1R3* as a query (e-value cut off < 0.01). The contigs with the best e-value were then aligned individually with each exon, using the Geneious software, and alignments were inspected by eye. Minor modifications were occasionally made, such as addition or subtraction of one or two codons to retain a splice-site signature. The extracted region for each exon was then verified by reciprocal BLASTN searches to the nr-database on NCBI.

Many of the bird sequences were incomplete: in cases where an exon spanned two contigs, "N"s were introduced to indicate a potential gap. If exon 1 was missing, a 5' start codon was added followed by "N"s to indicate a gap, to enable bioinformatic analyses to be performed. The two instances of possible in-frame stop codons were replaced with NNN for the same reason, and one instance of a frameshift-inducing insertion was also replaced by Ns. In cases where internal Ns were introduced, the subsequent sequence was checked to ensure that the correct frame was retained.

**Notes on T1R assembly**

In those cases where the sequence was incomplete, we cannot be certain that the gene is functional. There are many gaps in these assemblies, especially around *T1R1*, resulting in the loss of the terminal exons: for example, with the exception of the ground tit and collared flycatcher, none of the contigs containing *T1R1* had exon 1, or exon 2, and exon 6 often did not have full coverage in many species. In the case of both the Amazon parrot and the macaw *T1R3*s the splice-site signature on the 5' end of exon 5 was missing, potentially indicating alterative splicing, alternate splice-site signatures, or pseudogenization. Both the ground finch and the zebra finch *T1R1* each had an in-frame stop codon, but because they occurred at the end of an exon or contig this could be due to differences in splicing or sequence quality. Exon 5 in the peregrine falcon *T1R1* also lacked a 5' splice site and only partially aligned with the other sequences: in this case the poorly aligned region was replaced with Ns to indicate uncertainty in the sequence at this region. In the mallard *T1R3*, a 37-bp insertion in exon 6 causes a frame shift, so this insertion sequence was removed. In the zebra finch, *T1R3* appears to be at least partially duplicated; only the sequence with the higher coverage was used (exons 2-6 present).

**Alignment**

The final dataset consisted of 68 *T1R* genes: *T1R2* from 12 species (16 genes): 4 fish, 4 mammals, 4 non-avian reptiles; *T1R3* from 26 species (26 genes): 5 fish, 4 mammals, 4 non-avian reptiles, 13 birds; and *T1R1* from 26 species (26 genes): 5 fish, 4 mammals, 4 non-avian reptiles, 13 birds. Multiple datasets were assembled: dataset 1 contained all 68 *T1R*s and was rooted with the human class C GPCR Calcium-sensing receptor (*CASR*); dataset 2 contained all the *T1R1* genes rooted with the cloned hummingbird *T1R3*, and dataset 3 contained all *T1R3* genes rooted with the hummingbird *T1R1*. We aligned these datasets using the TranslatorX server (*29*) and the MAFFT alignment program (*30*). Before selecting MAFFT, we tested multiple alignment programs (MAFFT, MUSCLE (*31*), T-COFFEE (*32*) and PRANK (*33*)), since good alignment is essential to all subsequent analyses. We used the program NormD (*34*) to assess alignment quality: no one method consistently outperformed the other. With the program MetAl (*35*), we ran pairwise comparisons between the alignments. There was a larger discrepancy between methods for *T1R1* alignments than for *T1R3* as expected due to the frequency of *T1R1* truncation. Subsequent comparisons of tree topology and PAML results using the four alignments showed largely concordant results.

**Phylogenetic reconstruction**

Maximum likelihood phylogenetic trees were constructed using PHYML 3.0 (*36*) and JTT+I+G as the most appropriate model of evolution (determined *a priori* using ModelGenerator v0.85 (*37*) and evaluated with AIC). Figure 23 shows the tree built for the entire dataset; nodal

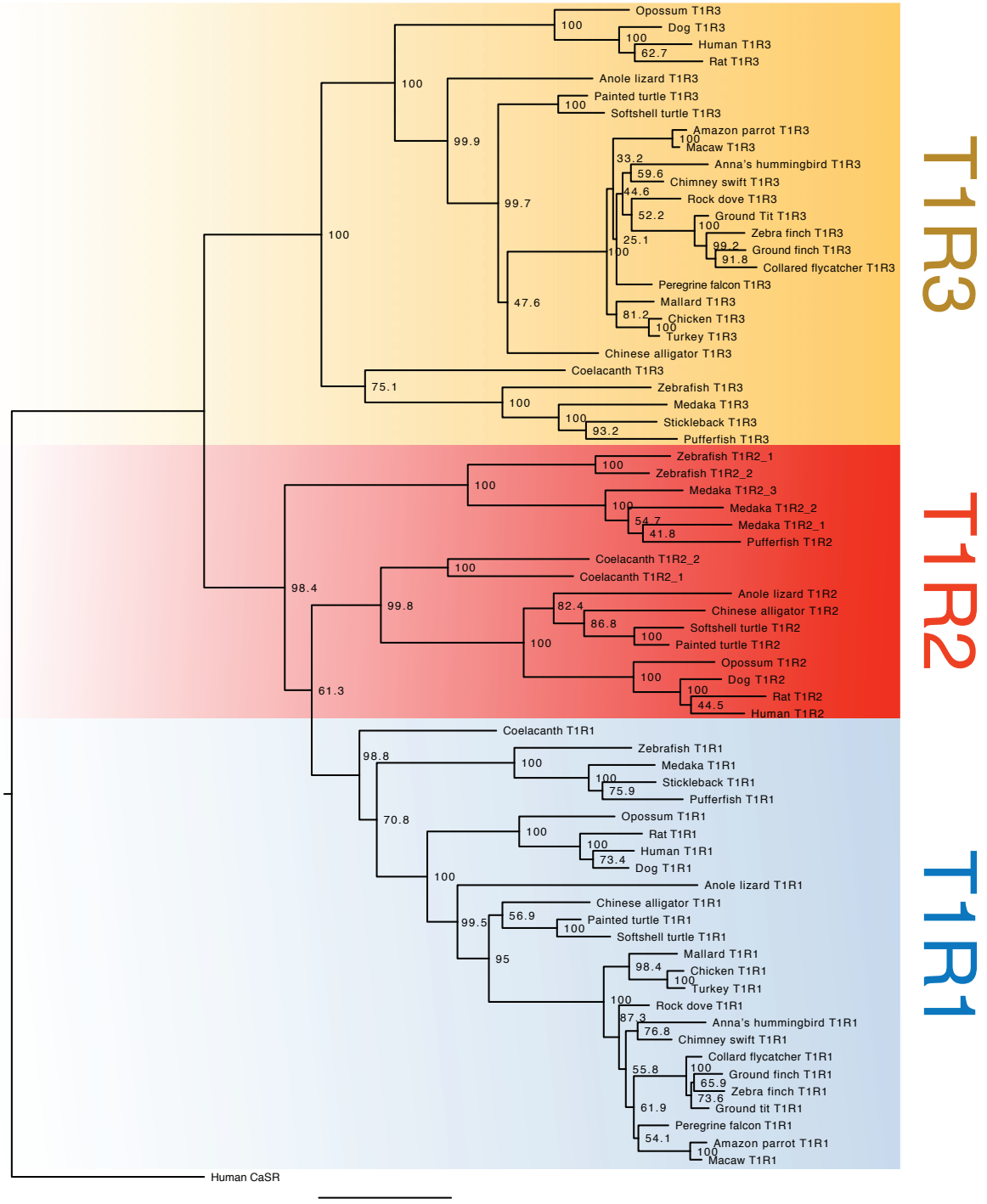



**Figure 23. Phylogenetic tree of T1Rs from birds and other vertebrates**. Maximum likelihood tree of 68 *T1R*s from 26 vertebrates was constructed and rooted using human Calcium Sensing Receptor (CaSR), scale bar = 0.4 substitutions per site.

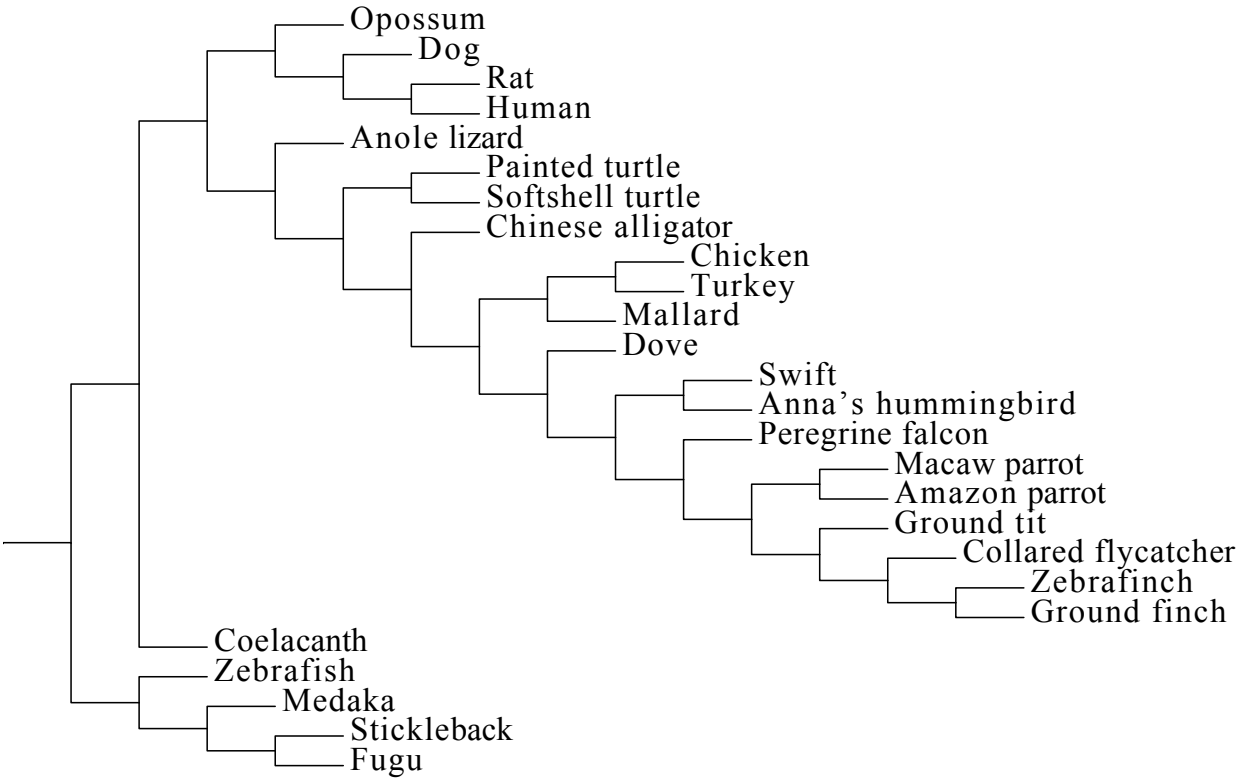



**Figure 24. Species tree used for phylogenetic analyses.** The PAML analyses were performed using the known species-tree topology, shown here.

supports were assessed with 1000 bootstrap replicates. Because of the occasional discordance between our gene-trees and the known species tree, as well as the low support obtained for many nodes (Figure 23), we created a tree (without branch lengths) which, to the best of our knowledge, corresponds with the known species tree (Figure 24); we used this tree for the subsequent analyses presented here. CodeML analyses appeared robust to minor topological discordances: when we compared the results from the PAML analyses with a gene-tree created from the MAFFT alignment, the results were largely similar.

**Tests of positive selection and selection pressure heterogeneity**

We used the program CodeML in the PAML package, version 4.4b (*38*), and tested multiple models using likelihood ratio tests (LRTs). Models applied under the CodeML framework follow standard nomenclature and are described in detail in Loughran *et al* (*39*). Four initial starting values of omega were tested for each model where omega is estimated from the data, and the omega value with the highest reproducible likelihood score was selected. If the four analyses had the same likelihood score, the initial value of omega = 0 was used unless otherwise stated. Seven types of likelihood ratio tests (LRTs) using $\chi^2$ tests of significance were run to compare alternative models: five estimating selection over the whole alignment and two branch-site models which focus on selection in the hummingbird, the swift, and the chicken lineages independently. For all analyses, we used alignments constructed with a single gene for *T1R1* and *T1R3* (datasets 2 and 3) and a tree that corresponded to the known species tree.

Results of CodeML analyses are shown in the Appendix. Both *T1R1* and *T1R3* showed signatures of heterogeneous selection pressures (M0 vs M3K2 and M3K2 vs M3K3). Positive selection over the whole gene was assessed by comparing Models 1 vs 2 and Models 7 vs 8; only the latter test was significant for *T1R1*, and the corresponding test against an additional null model (M8a) was also significant.

Branch-site models (*40*) and Bayes Empirical Bayes (BEB) analysis (*41*) were used to identify positive selection in specific lineages and at specific sites. In model 1 ("neutral model"), codons are allowed to evolve either neutrally ($\omega$ 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 ($\omega > 1$). The proportion of sites ($p_0$, $p_1$, $p_{2a}$, and $p_{2b}$) corresponding to each $\omega$ category,

and the associated ω values in each lineage are listed in the Appendix. 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, as described below. However as the additional proposed LRT with the null model for model A (*42*) was not significant we could not definitively rule out relaxed selection rather than positive selection in the hummingbird lineages with purifying selection in others with this test.

**Putatively selected sites**

To examine the significance of the sites identified in Model A as potentially under selection in the hummingbird, we located these residues on an alignment with other residues shown through mutational studies to play a role in ligand binding or receptor activation. The results are shown in Figure 25. In an exciting convergence of approaches, two sites independently detected by our chimeric analysis were also among the 6 sites identified by PAML. More sites are likely involved as well, and adding new sequences that refine the topology may improve predictions: a consistent seventh site was identified in tests using the T1R3 gene tree, and in tests where the outgroup was omitted.

Some of the BEB sites are close to residues of known importance in either *T1R1* or *T1R3* (see also the Appendix). The site with the highest posterior probability (0.985) lay outside

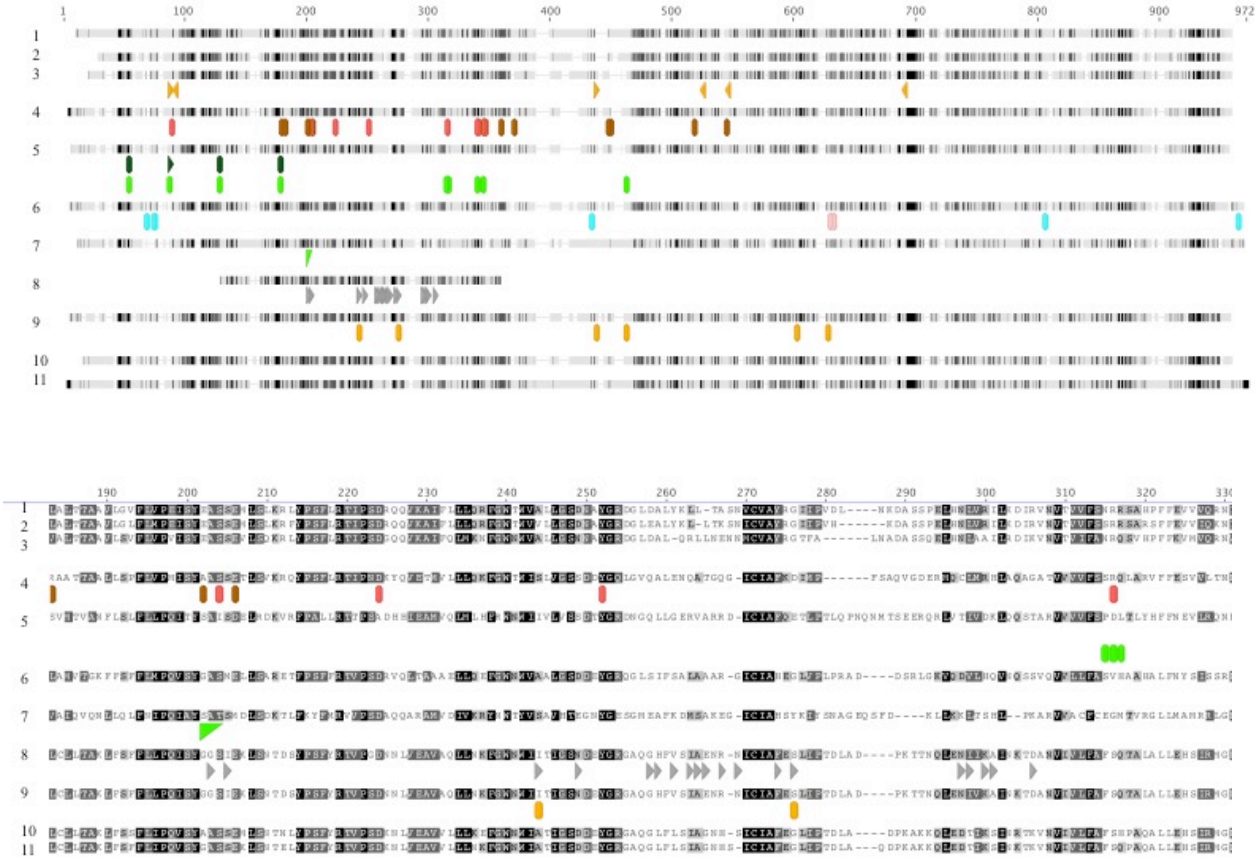


**Figure 25. Putatively selected sites in hummingbird *T1R*s. Top:** Summary of information from other studies regarding important sites. Sequence 1 = Ancestral *T1R1*, 2 = Swift *T1R1*, 3 = Hummingbird *T1R1*, 4 = Human *T1R1*, 5 = Human *T1R2*, 6 = Human *T1R3*, 7 = Human metabotropic glutamate receptor *GRM5*, 8 = Hummingbird *T1R3* (region with 19 identified sites), 9 = Hummingbird *T1R3*, 10 = Swift *T1R3*, 11 = Ancestral *T1R3*. BEB sites identified by the CodeML analyses are shown as yellow triangles (T1R1) or yellow ovals (T1R3). References: red sites from (*46*); brown are sites in (*15*); turquoise are from (*47*), dark green from (*48*), light green ovals from (*49*); light green triangle from (*11*), pink from (*5*), 19 non-consecutive key residues shown as grey triangles on the hummingbird *T1R3* sequence. **Bottom:** Close-up of the important region for the sweet response dissected in our chimeric analysis.

the region which we dissected in the chimeric work; intriguingly, it is a single residue away from an identified site in the human cysteine rich domain (CRD). This site is important for the response to brazzein as well as to other sweeteners, and is thought to be involved in inter-subunit coupling (*4*, *5*). It will be exciting to examine the functional role of this site, as well as sites identified in *T1R1*. Curiously, one predicted site in *T1R1* aligns with one predicted site in *T1R3*: analyses of intermolecular coevolution in these genes may also yield insights into coordinated changes necessary for the shift in function.

**Ancestral sequence reconstruction and future directions**

Using the program FASTML (*43*), we generated a putative ancestral reconstruction of *T1R3* and *T1R1* from datasets 1 and 2. For both genes, the ancestral sequences were predicted to be more similar to the swift than to the hummingbird (Figure 25): the pairwise similarity of hummingbird *T1R3* to the joint ancestral reconstruction at the swift-hummingbird common ancestor was 73.3%, whereas the swift- ancestral sequence was 82.3% similar (for *T1R1*, 76.0% and 85.4%, respectively). At the 19 key sites we identified, the ancestral sequence is predicted to be identical to the swift; similarly, in the 12 sites identified in the BEB analysis across the two genes, the predicted ancestral sequences are also identical to the swift sequences. Introducing mutations into the swift sequence, or, better, synthesizing these putative ancestral sequences and performing mutagenesis of candidate regions and selected sites in the ancestral background (*44*, *45*) will be an exciting avenue for future research, enabling perhaps identification of other key residues and also allowing investigation of the relative role and order of occurrence of key sites and the importance of epistasis between them. The shift from insectivory to nectarivory required immense morphological, behavioral and physiological changes in hummingbirds after they

diverged from a swift-like ancestor. Estimating the timing of changes at key residues will allow us to begin to ask questions about the role of this receptor in the change in taste perception and behavior, and the timing of substitutions relative to the diversification of the hummingbird radiation. Was the shift in perception a key adaptation to a new diet? Did it occur before or after the divergence of major groups of hummingbirds? Understanding the sequence of events and comparing changes in other nectar-feeding birds will inform our understanding of the causes and the consequences of this dramatic change in lifestyle and feeding ecology in this lineage.

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## Chapter 3

## Bi-functional hummingbird taste receptor guides amino acid preferences

### Introduction

Hummingbirds detect carbohydrates by extensive modification of their amino acid (umami) taste receptor, a unique recovery of a lost function after the disappearance of the putative ancestral sweet receptor early in bird evolution (*1*). However, insects nevertheless make up a large part of the diet of hummingbirds, and amino acids occur in nectar; yet whether hummingbirds still perceive the taste of amino acids is unknown. Previous behavioral studies indicated that many amino acids were either not detected, or aversive. We compared the responses of umami receptors (T1R1/T1R3) from hummingbirds, chickens and swift in cell-based assays to a panel of amino acids. Here, we show that in taste trials with wild hummingbirds, agonists identified in cell culture elicit preference behavior similar to that seen to nectar sugars. This indicates that the hummingbird T1R1/T1R3 is a bi-functional receptor and raises the possibility that in hummingbirds, sweet and umami may be detected by the same receptor as a single, pleasurable stimulus.

Surprisingly little is known about bird taste preferences and the underlying genetics. Until recently, the importance of chemosensory detection in birds has largely been ignored, and differences from mammalian perception have not been widely explored. Yet important variation in chemoreception exists among major clades of vertebrates. For instance, capsaicin, which elicits an aversive response in mammals, is not detected by birds (*2*, *3*); birds thus are able to ingest the fruits of pepper plants and are important seed dispersers (*4*, *5*). In another difference from work in mammalian model systems, our recent findings show that hummingbirds lack the

sweet receptor yet detect carbohydrates via their remodeled umami receptor. Lineage-specific differences exist and are the product of ecological pressures as well as evolutionary history.

The taste of umami, one of the five basic tastes, has been studied both molecularly and behaviorally in mammals and also in fish, but data outside these groups are rare. In mammals, umami (which means savory in Japanese) is the taste of amino acids, and is mediated by a heterodimer of two Class C G-Protein Coupled Receptors (GPCRS) in the same Taste Receptor Type I (*T1R*) family (*6*). Both genes are required for taste perception, and knock-out mice lacking either *T1R1* or *T1R3* are umami-insensitive (*7*). Some species have broadly-tuned receptors: in mice, this receptor pair is activated by a wide range of amino acids (*6*), but in certain old-world primates, the umami receptor has evolved to be narrowly-tuned to recognize two acidic amino acids, glutamate and aspartate (*8*). Our human receptor is activated most strongly by glutamate, a common (and maligned) culinary additive in the form of MSG (monosodium glutamate). While key residues involved in receptor specificity have been mapped (*8*), the ecological and dietary reasons behind the receptor preferences are still unknown.

Few behavioral studies of bird umami preference have been performed. Birds show compensatory feeding for carbohydrates (*9*) and for amino acids (*10*), so experiments which are not brief-access may potentially reflect post-ingestive effects instead of indicating pure sensory responses. In taste trials with two songbird species, red-winged blackbirds (*Agelaius phoeniceu*s) and European starlings (*Sturnus vulgaris*), animals were given 2-bottle tests and consumption was compared over 30-minute intervals. Concentrations of 0.1%, 0.4%, 0.7% and 1% (w/v) L-alanine were tested (11-112 mM), and while differences in preferences existed between sexes, a preference was found for increasing concentrations (*11*). Studies with other species and different amino acids show mixed responses: quail and chickens show no preference for MSG at some

concentrations and aversion at others (*12*), and preference for methionine relies on nutritional state and learned visual cues (*10*).

Many nectar-feeders such as hummingbirds, some honeyeaters, and lorikeets extract the majority of their energy from the carbohydrates in plant nectar (*13*), and supplement their diet with insects and pollen. Nectar was initially thought to be a simple mix of carbohydrates, but in the 1970s, the discovery was made that many nectars also contain amino acids, and that the composition and concentration of amino acids present varied by pollinator type (*14-16*). In a study by Hainsworth and Wolf in 1976, taste preferences of hummingbirds were tested in response to a panel of amino acids reported in nectar (*17*): concentrations similar to ~0.786 mm and 25.14 mM histidine were added to sucrose solutions and were presented to hummingbirds in two-bottle tests. At these concentrations, approximately equal to or double those of amino acids reported in nectar, no birds displayed a preference. Variability existed among the 6 individuals tested, but at the higher concentration, all but glycine, threonine, proline and alanine were avoided. Here, again trials were over extended periods of time, lasting from 3-6 hours.

**Methods and Results**

Our early experiments had tested amino acids in two previous scenarios: experiments with black-chinned hummingbirds in 2008 tested 2 M L-proline, because to mammals, proline is an amino acids that is 0.25 as sweet as sucrose (*18*)—at this concentration, birds avoided proline as well as mixtures of proline and sucrose (Chapter 1). In later experiments with Anna's hummingbirds, we tested L-alanine, since this was one of the two amino acids we found to

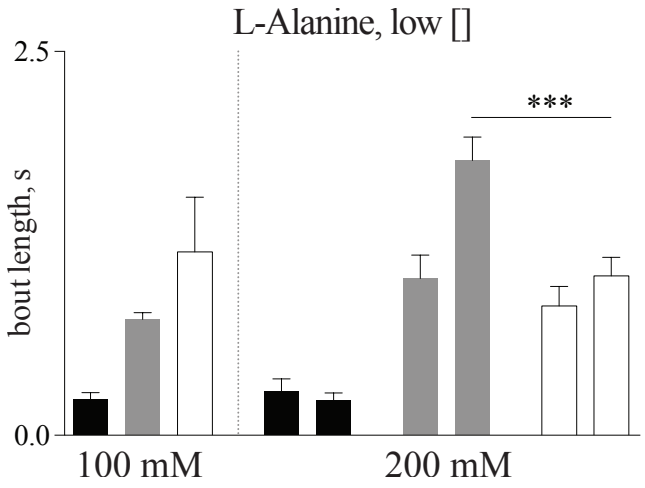


**Figure 26. Hummingbirds show no preference for low concentrations of L-alanine.** L-alanine at 100 and 200 mM did not elicit the long bouts characteristic of preference behavior towards appetitive solutions. Black bars = L-alanine, grey = mix of L-alanine and sucrose, white = sucrose 500 mM. For 100mM trial, Anna's males were scored, for 200 mM both Anna's males (left bars) and all birds (right bars) were scored. Among the broader population of birds we saw an increase in bout length at feeders containing a mixture of L-alanine and sucrose (Kolmogorov-Smirnov tests for differences between mixture/sucrose:***$p \leq 0.001$).

activate the receptors in the cell-based assay. Data from Baker and Baker indicated that amino acids in nectar were present merely at low concentrations (25 mM), although subsequent work has shown that some South African flowers pollinated by sunbirds (Nectariniidae; passerine nectar-feeding birds, not close relatives of hummingbirds) have nectars with over 100 mM amino acids (*19*). Thus, to approximate ecologically-relevant stimuli, we used lower concentrations than for carbohydrates and tested 100 mM and 200 mM (Figure 26). In these trials, L-alanine was tested in the presence of a 500 mM sucrose control, and birds did not exhibit a preference for solutions containing purely L-alanine alone. However, at 200 mM, the mix of sucrose and L-

alanine elicited higher bout lengths when all birds were examined, indicating perhaps an enhanced palatability. Although this assay is intended primarily to just discriminate between preference and rejection, this result suggested a potential response. Two alternative scenarios could explain these patterns: first, L-alanine was not sweet alone but potentiated the response to sucrose—analogous to the potentiation of the glutamate response by IMP in some mammals (*6*). Alternatively, amino acids such as L-alanine may be appetitive alone, but not at this concentration—or not when presented simultaneously with a 500 mM sucrose feeder.

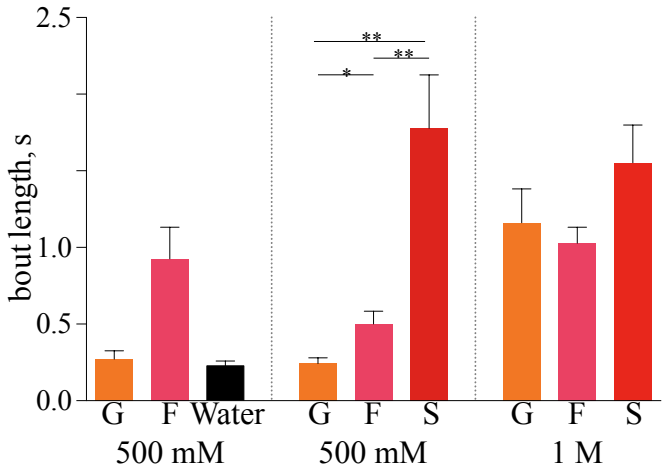


**Figure 27. Context and concentration matter for hummingbird taste preferences**. Fructose (F) and glucose (G) did not elicit long preference bouts at 500 mM but at 1 M were consumed with equal avidity as solutions containing sucrose (S) (Kolmogorov-Smirnov tests for differences between concentrations: *p≤0.05, **p≤0.01).

We had previously seen that responses of hummingbird *T1R* taste receptors to amino acids in cell culture were of about the same magnitude as responses to glucose (*1*). This indicated that perhaps we were using concentrations that were too low to evoke preference behavior,

because in our previous experiments, we saw that glucose was only appetitive to the birds in behavioral tests at very high concentrations (Figure 27): even 500 mM glucose (a higher concentration than any amino acid we tested) was not preferred. Interestingly, a recent study by Medina-Tapia *et al* (*20*) demonstrated far lower gustatory thresholds for all three carbohydrates in broad-billed hummingbirds (*Cynanthus latirostris*), indicating the important difference between detection and preference and highlighting as well the importance of context (i.e. which other stimuli are presented in concert).

Concentration is a critical factor for selection of carbohydrates. Many studies have focused on concentration-dependent changes in preferences of nectar-feeders, which appear to be related to activities of digestive enzymes: for example, a shift from preference for an equicaloric mixture of hexoses (glucose and fructose) compared to sucrose at low concentrations to a sucrose solution at higher concentrations has been observed in some species and is attributed to assimilation rates of digestive enzymes (*21*, *22*). Our behavioral tests are not directly comparable to these studies because equimolar rather than equicaloric solutions are presented (*23*). However, in our study, concentration matters as well: glucose was not preferred at 500 mM when presented simultaneously with fructose or sucrose, but at 1 M, it elicited long bouts (Figure 27).

Thus, if the same taste receptor was responsible for detection of amino acids as well as glucose, it then seemed possible that high concentrations (even if not ecologically relevant) might activate the receptor, although it was also likely that, like sweeteners, these concentrations might have off-tastes as well. The pilot trial of 2 M L-proline was markedly aversive and caused reduction in consumption when mixed with 500 mM sucrose, suggesting that this might occur.

Therefore, in the subsequent field season, we tested high concentrations of amino acids, presented in triplicate simultaneously with water, not sucrose. 1 M L-alanine, L-serine and MSG, and 2 M L-serine and MSG were tested. MSG was rejected, but birds responded positively to L-alanine and L-serine, drinking with longer bouts than from paired water feeders (Figure 28). The response to amino acids was striking: preference behavior was elicited, without beak-removal or hesitation, mirroring behavior elicited by feeders containing carbohydrates. This was unexpected, since otherwise normally non-aversive stimuli, such as water, often cause headshaking and beak-withdrawal behaviors: the behavioral reaction suggests that these preferred stimuli may indeed be indistinguishable.

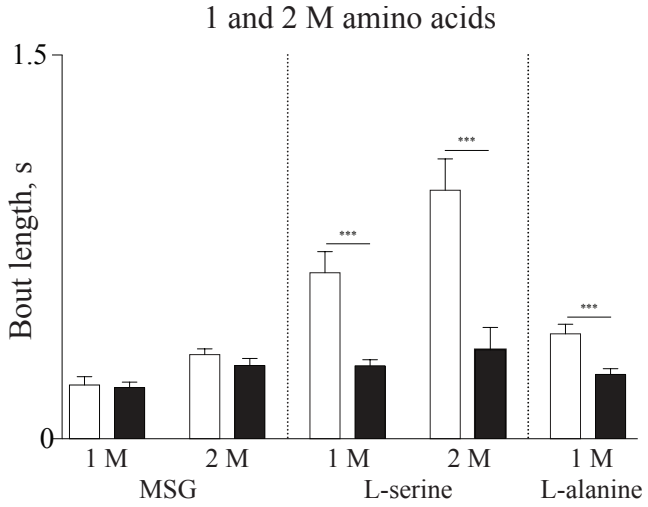


**Figure 28. Hummingbirds prefer high concentrations of L-alanine and L-serine, but not MSG.** Presentation of L-alanine and L-serine at 1 M concentrations elicited preference behavior. Increasing the concentration from 1 M to 2 M resulted in longer bouts from L-serine, but not from MSG (Kolmogorov-Smirnov tests for differences between stimuli/water: ***$p \leq 0.001$). Black bars = water, white bars = amino acids. Data shown are from all birds (sexes and species pooled, samples sizes in the Appendix).

We decided to further probe the behavioral response to amino acids. Dr. Toda at the University of Tokyo tested the responses of the T1R1/T1R3 hummingbird, chicken, and swift receptors cloned previously (Chapter 2) to a panel of 17 amino acids, or subsets thereof, at 50 mM, 100 mM and 200 mM. We then selected the amino acids that could easily go into solution at high enough concentrations that might elicit preference in our behavioral assay. We chose the three amino acids that activated the hummingbird receptor (L-alanine and L-serine, as tested previously, and also glycine), two that did not activate the receptor (L-proline, L-valine), as well as L-threonine, which had only a slightly larger response than L-proline.

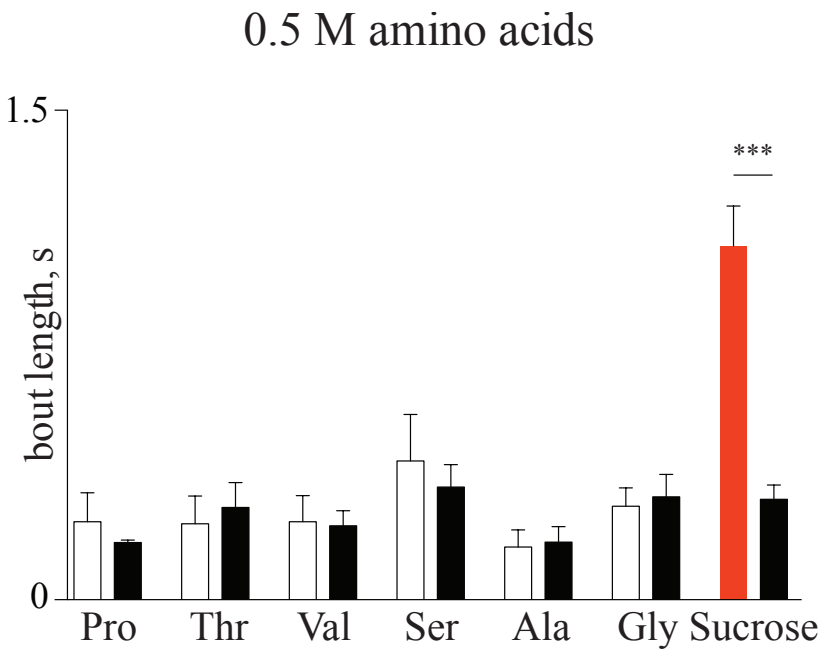


**Figure 29. Low concentrations of amino acids are not preferred by hummingbirds.** Sucrose (500 mM, red bar) elicits long drinking bouts; amino acids at 500 mM concentrations (white bars) do not. Stimuli were presented separately, with water as a control. Occasional long bouts (>1 sec) were observed to 500 mM L-serine, but the overall distribution of bout lengths did not significantly differ from bout lengths at feeders containing water (black bars) (Kolmogorov-Smirnov tests for differences between stimuli/water, ***$p \leq 0.001$).

Subsets of these amino acids were tested at concentrations ranging from 500 mM to 2 M at the same location where prior behavioral tests were conducted, in the Santa Monica Mountains. Perhaps because of seasonal differences (fewer individuals, higher intensity of territoriality, larger numbers of hornets which chased off hummingbirds) the numbers of birds that visited the feeders was far fewer. Most birds were Anna's hummingbirds, although occasional black-chinned hummingbirds and Allen's hummingbirds visited the feeders as well.

While low concentrations did not spur significantly longer visits than the paired water feeders (Figure 29), at higher concentrations, birds responded positively to the amino acids that activated the receptor (Figure 30). As before, a similarly unhesitating preference was exhibited to receptor agonists, but long bouts were never observed at any concentration of L-proline, which did not activate the receptor (Figure 30).

**Evidence of possible amino acid imbalance**

After presentation of higher concentrations of L-alanine and glycine, some indication of aberrant flight behavior was noted, and in some visits, birds took long drinks from water, which otherwise only occurred rarely. Thus, tests with high concentrations of these stimuli were discontinued because of potential toxicity. Hainsworth noted that after 3-4 hours of exposure to glycine, hummingbirds stopped feeding from feeders and started catching insects (*17*); he cites studies from rats where amino acid imbalance resulted in feeding changes and suggests something similar might be occurring. Weischer, in a paper in German in 1965, presented lizards (two species in the genus *Lacerta*) with L-alanine and Glykokoll (glycine) and described unexpected paralysis in some individuals (*24*). The physiological effects are interesting but

beyond the intent of our study; we recommend that future trials with amino acids in birds and reptiles carefully monitor physiological and neurological parameters.

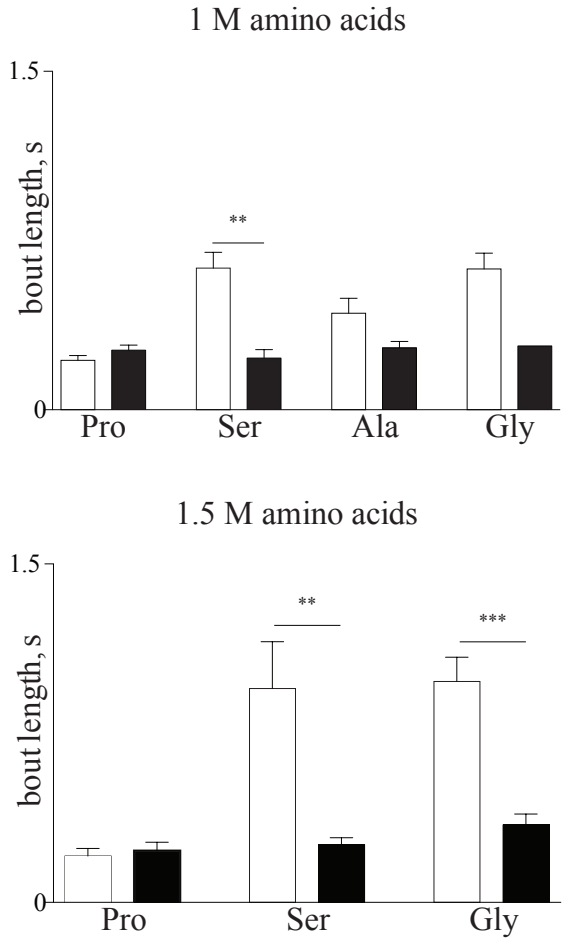


**Figure 30. Glycine is appetitive to wild hummingbirds**. Hummingbirds exhibit appetitive behavior towards glycine, a T1R1/T1R3 receptor agonist, but not towards L-proline, which does not activate the receptor. White bars = amino acids, black bars = water (Kolmogorov-Smirnov tests for differences between stimuli/water: **$p \leq 0.01$, ***$p \leq 0.001$).

## Discussion

The appetitive response of hummingbirds to amino acids raises ecological questions as well as questions about taste processing and the evolution of neural circuits. The concordance

between the responses in cell culture and the birds' preferences in behavioral tests lends support to the role of the T1R1/T1R3 heterodimer as a repurposed yet still bi-functional receptor. The responses to amino acids might not be shaped by the diet of extant hummingbirds, but may represent remnants of the ancestral function of this gene.

Amino acids are present in nectar, where they may well play an important role in enhancing palatability, either because of their intrinsic attractive value, or because they synergistically increase the hedonic value of carbohydrates. Amino acids in nectar could perhaps represent an underappreciated source of nitrogen or energy for these birds; however, bird-pollinated plants have on average low levels of amino acids, much lower than plants visited by other pollinators, such as dung-flies (*14*). One possibility is that nectars might exist that have high levels of amino acids but that have not been surveyed. Recent work on certain South African species reveals higher amino acid concentrations, for instance over 100 mM, in some plants pollinated by birds (*19*).

Even with mammals, however, adaptive causes behind umami receptor tuning are still unclear: mice, fish and some primates have broadly-tuned receptors, while humans and other old world monkeys have narrowly-tuned receptors that respond most strongly to acidic amino acids such as glutamate. Understanding forces shaping receptor tuning will greatly contribute to our understanding of these genes, their role in nutrient sensing and perhaps in other extra-oral functions as well.

The hummingbird T1R1/T1R3 response profile differs from that of swifts. Swift and chicken receptors respond in similar ways (Dr. Toda, pers, comm); both, for instance respond to arginine and histidine, two basic amino acids, that do not activate the hummingbird receptor. Conversely, the response to glycine is unique to hummingbirds. Because chickens and swifts eat

insect (swifts exclusively so, chickens are omnivores), this might reflect a physiological or dietary role. Yet, similar to results in other species, data on dietary composition does not clearly match receptor tuning. A study on the diet of an Australian possum (*25*) breaks down the amino acid make-up of insect hemolymph and pollen—the amino acid with the highest concentration in hemolymph is proline, which is not an agonist for any bird T1R1/T1R3; like with the other vertebrate umami receptors, no clear answer emerges. In a study of amino acids in nectars of plants with many different pollinators, Baker and Baker list the amino acids with the highest frequency of occurrence as alanine, arginine, serine, proline and glycine (from 95-84% of 395 species analyzed) (*26*). Hainsworth and Clark list the six most common amino acids in hummingbird pollinated flowers as proline, serine, threonine, glycine, alanine and arginine (cited from Baker and Baker as pers, comm (*17*)). Glycine is an essential amino acids for birds (*27*), unlike for mammals. However, while detection of glycine might be adaptive and in some manner physiologically beneficial, it might merely be a historical consequence of the structure of the molecule and activation might be a secondary by-product of changes that allowed carbohydrates to be detected. Interestingly, glycine also tastes sweet to mammals (*18*), thus a similar binding pocket might result in detection of both chemicals.

Even if amino acid concentrations in hummingbird-pollinated flowers are present at sub-threshold levels too low to be detected if they were the only compound present, they might have a synergistic or potentiating effect by enhancing the response to nectar sugars. This phenomenon occurs in ants: the larvae of lycaenid butterflies secrete droplets of sugar mixed with glycine. Tasteless alone, glycine acts synergistically by enhancing the response of the attendant host ants, but not other species, to specific carbohydrates (*28*, *29*). Invertebrates detect sugars with convergently-evolved and non-homologous gustatory receptors, but synergism and potentiation

is documented for vertebrate taste receptors as well. This is not surprising, as T1Rs are large complicated proteins with multiple sites where ligands interact. The ribonucleotide inosine monophosphate (IMP) is tasteless to mice and humans but potentiates the umami response in mammals (*6*, *30*), and the artificial sweetener cyclamate potentiates the T1R1/T1R3 heterodimer in cell culture (*31*). Nectar is a complex mixture (*32*, *33*) and it will be interesting to examine a role for possible potentiation by diverse nectar constituents on hummingbird taste receptor responses.

In mammals, taste information is processed by 'labeled lines' (*34*, *35*)— the identity of the taste receptor cell, not the ligand itself, determines tastant quality. For instance, multiple bitter receptors detect a wide range of chemicals, but the receptors are expressed in a single cell type and yield a single, aversive taste. If amino acids and sugars are detected by the same receptor pair expressed in the same cells, it seems likely that these stimuli might be indistinguishable to the taster, and instead of being perceived as 'sweet' or 'savory', they might both elicit a single pleasurable sensation. The higher-level consequences and central processing of these stimuli will be exciting to examine, as will the question of whether convergently-evolved nectarivorous birds detect and perceive amino acids in a similar fashion. Once new technologies allow for genome-editing, labeling, and tracing of taste-related neurons, comparative studies of taste in birds will provide valuable insights into vertebrate taste biology and the evolution of sensory perception.

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## Appendix

Table S1

Number of visits of black-chinned hummingbirds in behavioral trials (for Figure 6 and 7A-C)

| Type | Stimulus | # visits | | |
|---|---|---|---|---|
| | | Stimulus | Mix | Sucrose |
| Amino acid | L-Proline | 39 | 44 | 48 |
| Amino acid | D-Asparagine | 23 | 53 | 62 |
| Amino acid | D-Tryptophan | 39 | 28 | 54 |
| Amino acid | D-Phenylalanine | 20 | 37 | 41 |
| Synthetic Sweetener | Cyclamate | 61 | 84 | 151 |
| Synthetic Sweetener | Sucralose | 45 | 94 | 89 |
| Synthetic Sweetener | Acesulfame K | 40 | 51 | 56 |
| Sugar Alcohol | Xylitol | 47 | 60 | 51 |
| Sugar Alcohol | Erythritol | 50 | 60 | 50 |
| Sugar Alcohol | Sorbitol | 43 | 59 | 55 |

Table S2

Number of visits of black-chinned hummingbirds in behavioral trials with different concentrations of sucrose (Figure 7D)

| | 0.25 | 0.5 | 1 | 1.5 | 2 | 2.5 |
|---|---|---|---|---|---|---|
| Sucrose (M) | 25 | 29 | 29 | 29 | 27 | 43 |

Table S3

Number of visits of wild hummingbirds in behavioral trials with carbohydrates and sugar alcohols (Figures 11 B and C)

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

Table S4

Number of visits of wild hummingbirds in behavioral trials with synthetic sweeteners (Figures 11B and 12)

| | # visits | | | | | |
|---|---|---|---|---|---|---|
| | # visits of Anna's males in 15 minute trial | | | # visits of all birds (first rotation only) | | |
| Stimulus | 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

Table S5

Number of visits of wild hummingbirds in behavioral trials (Figure 13A and B)

| | # visits | | | | | |
|---|---|---|---|---|---|---|
| | # visits of Anna's males in 15 minute trial | | | # visits of all birds (first rotation only) | | |
| Stimulus | Stimulus | Mix | Sucrose | Stimulus | Mix | Sucrose |
| 5 mM Sucralose 2013 | 21 | 65 | 31 | 28 | 68 | 103 |
| 5 mM Sucralose 2014, replicate 1 | 10 | 51 | 32 | | | |
| 5 mM Sucralose 2014, replicate 2 | 17 | 48 | 29 | | | |
| 5 mM Sucralose 2014, replicate 3 | 10 | 27 | 36 | | | |

Table S6

Number of visits of wild hummingbirds in behavioral trials (Figures 13C and 14A)

| | # visits | | | |
|---|---|---|---|---|
| Trial | # visits Anna's males | | # visits of all birds | |
| | 1 | 2 | 1 | 2 |
| 1 = 5 mM sucralose<br>2 = Water | 21 | 22 | 58 | 46 |
| Splenda ®<br>1 = 0.55 mM<br>2 = 1.38 mM | 15 | 7 | 55 | 41 |

Table S7

Number of visits of wild hummingbirds in behavioral trials (Figure 14B)

| | # visits | | | |
|---|---|---|---|---|
| Trial | # visits all males | | # visits of all females | |
| | 1 | 2 | 1 | 2 |
| Splenda ®<br>1 = 2.22 mM<br>2 = Water | 57 | 54 | 105 | 93 |

Table S8

Number of visits of wild hummingbirds in behavioral trials (Figure 26)

| | # visits | | | | | |
|---|---|---|---|---|---|---|
| | # visits of Anna's males | | | # visits all birds | | |
| Stimulus | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 = 100 mM L-Alanine<br>2 = Mix<br>3 = 500 mM Sucrose | 5 | 51 | 8 | | | |
| 1 = 200 mM L-Alanine<br>2 = Mix<br>3 = 500 mM Sucrose | 2 | 18 | 17 | 19 | 48 | 43 |

Table S9

Number of visits of wild hummingbirds in carbohydrate behavioral trials (Figure 11D and Figure 27)

| | # visits (Anna's males only) | | |
|---|---|---|---|
| Stimulus | 1 | 2 | 3 |
| 1 = 500 mM Glucose<br>2 = 500 mM Fructose<br>3 = Water | 8 | 23 | 11 |
| 1 = 500 mM Glucose<br>2 = 500 mM Fructose<br>3 = 500 mM Sucrose | 16 | 39 | 26 |
| 1 = 1 M Glucose<br>2 = 1 M Fructose<br>3 = 1 M Sucrose | 19 | 70 | 16 |

Table S10

Number of visits of wild hummingbirds (all birds) in amino-acid behavioral trials (Figure 28)

| | 1 M | Water | 2 M | Water |
|---|---|---|---|---|
| MSG | 28 | 24 | 52 | 43 |
| L-Serine* | 68 | 34 | 84 | 33 |
| L-Alanine* | 55 | 43 | | |

*for 1M trial, first ~100 bouts scored

Table S11

Number of visits of wild hummingbirds (all birds) in amino-acid behavioral trials (Figures 29 and 30)

| | 0.5 M | Water | 1 M | Water | 1.5 M | Water |
|---|---|---|---|---|---|---|
| L-Threonine | 3 | 6 | | | | |
| L-Valine | 9 | 7 | | | | |
| L-Proline | 3 | 2 | 31 | 30 | 12 | 17 |
| L-Serine | 12 | 12 | 21 | 11 | 43 | 12 |
| L-Glycine | 14 | 9 | 31 | 1 | 50 | 24 |
| L-Alanine | 3 | 5 | 25 | 17 | | |
| Sucrose | 76 | 33 | | | | |

**Table S12. Downloaded genomes**

| Common Name | Scientific Name | Database | Date | Number |
|---|---|---|---|---|
| Collared Flycatcher | *Ficedula albicollis* | NCBI | 11/1/2103 | AGTO02.fsa.1/2/3 |
| Peregrine Falcon | *Falco peregrinus* | NCBI | 11/1/2103 | AKMT01.fsa.1/2 |
| Tibetan Ground Tit | *Pseudopodoces humilis* | NCBI | 11/1/2103 | ANZD01.fsa.1/2 |
| Mallard | *Anas platyrhynchos* | NCBI | 11/1/2103 | ADON01.1/2/3 |
| Rock Dove | *Columba livia* | NCBI | 11/1/2103 | AKCR01.fsa.1/2 |
| Medium Ground Finch | *Geospiza fortis* | NCBI | 11/1/2103 | AKZB01.fsa.1/2 |
| Scarlet Macaw | *Ara macao* | NCBI | 11/1/2103 | AMXX01.fsa.1/2/3 |
| Puerto Rican Amazon | *Amazona vittata* | NCBI | 11/1/2103 | AOCU01.fsa.1/2 |
| Wild Turkey | *Meleagris gallopavo* | NCBI | 11/1/2103 | ADDD01.fsa.1/2 |
| Zebra Finch | *Taeniopygia guttata* | NCBI | 11/1/2103 | ABQF01.fsa.1/2/3/4 |
| Chinese Alligator | *Alligator sinensis* | NCBI | 9/29/2103 | AVPB01.fsa.1/2/3/4/5 |

**Table S13: Paramters for PAML analyses for each gene, and Likelihood Ratio Tests (LRTs) comparing models**

**(a) Parameter estimates from CODEML for T1R3**

| Model | P | Estimates of parameters | Positively selected sites |
|---|---|---|---|
| M0 : one ratio | 1 | w = 0.21535 | None |
| **Site-specific:** | | | |
| M1:Neutral | 2 | $p_0$ = 0.66992, $p_1$ = 0.33008, $w_0$ = 0.16346, $w_1$ = 1 | Not allowed |
| M2:Selection ($\omega_i$=1) | 4 | $p_0$ = 0.66992, $p_1$= 0.10516, $p_2$ = 0.22492, $w_0$ = 0.16346, $w_1$ = 1, $w_2$ = 1 | BEB 1 > 0.5 |
| M3:Discrete(K = 2) | 3 | $p_0$ = 0.45877, $p_1$ = 0.54123, $w_0$ = 0.07289, $w_1$= 0.39724 | None |
| M3:Discrete(K = 3) | 5 | $p_0$ = 0.21907, $p_1$ = 0.42624, $p_2$ = 0.35469, $w_0$ = 0.02610, $w_1$ = 0.16888, $w_2$ = 0.50869 | None |
| M7: Beta | 2 | p = 0.80374, q = 2.22716 | Not allowed |
| M8: Beta&Omega > 1 | 4 | $p_0$ = 0.98906, p = 0.82221, q = 2.36064, ($p_1$= 0.01094), w = 1 | BEB 6 > 0.5 |
| M8a: Beta&Omega = 1 | 3 | $p_0$ = 0.98906, p = 0.82221, q = 2.36064 ($p_1$ = 0.01094 ), w = 1 | Not allowed |
| **Branch-specific: Hummingbird** | | | |
| Model A | 4 | $p_0$ = 0.62945, $p_1$ = 0.30923 $p_{2a}$ = 0.04112, $p_{2b}$ = 0.02020, $w_0$ = 0.16157 $w_1$ = 1, $w_2$ = 2.42172 | BEB 6 > 0.5 (1 > 0.95) |
| Model A null | 3 | $p_0$ = 0.58620, $p_1$ = 0.28855, $p_2$ = 0.08393, $p_{2b}$ = 0.04132, $w_0$ = 0.16118, $w_1$ = 1, $w_2$ = 1 | Not allowed |
| **Branch-specific: Swift** | | | |
| Model A | 4 | $p_0$ = 0.66178, $p_1$ = 0.32520 $p_{2a}$ = 0.00873, $p_{2b}$ = 0.00429, $w_0$ = 0.16288 $w_1$ = 1, $w_2$ = 6.95688 | BEB 3 > 0.5 |
| Model A null | 3 | $p_0$ = 0.61711, $p_1$ = 0.30374, $p_{2a}$ = 0.05305 , $p_{2b}$ = 0.02611, $w_0$ = 0.16252, $w_1$ = 1, $w_2$ = 1 | Not allowed |
| **Branch-specific: Chicken** | | | |
| Model A | 4 | $p_0$ = 0.66992, $p_1$ = 0.33008 $p_{2a}$ = 0, $p_{2b}$ = 0, $w_0$ = 0.16346 $w_1$ = 1, $w_2$ = 1 | None |
| Model A null | 3 | $p_0$ = 0.66992, $p_1$ =0.33008, $p_{2a}$ = 0, $p_{2b}$ = 0, $w_0$ = 0.16346, $w_1$ = 1, $w_2$ = 1 | Not allowed |

## Likelihood Ratio Tests for T1R3:

| Comparison | Null Model lnL | Alt Model lnL | *df* | Adjusted deltaL | Critical Value | Significant? |
|---|---|---|---|---|---|---|
| ***Site Analysis*** | | | | | | |
| m0 v m3Discrtk2 | -47285.006233 | 46212.386311 | 2 | 2145.239844 | 5.99 | YES |
| m3Discrtk2 v m3Discrtk3 | -46212.386311 | -46057.02453 | - | 310.723556 | 1 | YES |
| m1Neutral v m2Selection ($\omega_i$=1) | -46589.49762 | -46589.49762 | 2 | 0 | 5.99 | NO |
| m7 v m8 | -46027.745424 | -46027.345046 | 2 | 0 | 5.99 | NO |
| m8a v m8 | -46027.345046 | -46027.345046 | 1 | 0 | 2.71 | NO |
| ***Branch Analysis*** | | | | | | |
| ***Hummingbird:*** | | | | | | |
| ModelA v m1Neutral | -46583.064803 | -46589.49762 | 2 | 12.865638 | 5.99 | YES |
| ModelA v ModelAnull | -46583.064803 | -46583.707922 | 1 | 1.286238 | 3.84 | NO |
| ***Swift:*** | | | | | | |
| ModelA v m1Neutral | -46586.767403 | 46589.49762 | 2 | 5.460438 | 5.99 | NO |
| ModelA v ModelAnull | -46586.767403 | -46587.645033 | 1 | 1.75526 | 3.84 | NO |
| ***Chicken:*** | | | | | | |
| ModelA v m1Neutral | -46589.497622 | 46589.497622 | 2 | 0 | 5.99 | NO |
| ModelA v ModelAnull | -46589.497622 | -46589.497622 | 1 | 0 | 3.84 | NO |

## (b) Parameter estimates from CODEML for T1R1

| Model | P | Estimates of parameters | Positively selected sites |
|---|---|---|---|
| M0 : one ratio | 1 | w = 0.22149 | None |
| **Site-specific:** | | | |
| M1:Neutral | 2 | $p_0$ = 0.65110, $p_1$ = 0.34890, $w_0$ = 0.14688 $w_1$ = 1 | Not allowed |
| M2:Selection | 4 | $p_0$ = 0.65110, $p_1$ = 0.10133 $p_2$ = 0.24757, $w_0$ = 0.14688, $w_1$ = 1, $w_2$ =1 | BEB 8 > 0.5 (1 > 0.95) |
| M3:Discrete(K = 2) | 3 | $p_0$ = 0.48295, $p_1$ = 0.51705 $w_0$ = 0.07265, $w_1$ = 0.44236 | None |
| M3:Discrete(K = 3) ($\omega_i$=1) | 5 | $p_0$ = 0.30702, $p_1$ = 0.39292, $p_2$ = 0.30006 $w_0$ = 0.03912, $w_1$ = 0.20915, $w_2$ = 0.62905 | None |
| M7: Beta | 2 | p = 0.68595, q = 1.70469 | Not allowed |
| M8: Beta&Omega > 1 | 4 | $p_0$ = 0.98685, p = 0.71953 , q = 1.90386 ($p_1$= 0.01315), w = 2.05591 | BEB 12 > 0.5 (2 > 0.95) |
| M8a: Beta&Omega = 1 | 3 | $p_0$ = 0.94352, p = 0.76784, q = 2.31952 ($p_1$ = 0.05648 ), w = 1 | Not allowed |
| **Branch-specific: Hummingbird** | | | |
| Model A | 4 | $p_0$ = 0.63900, $p_1$ = 0.34149 $p_{2a}$ = 0.01272, $p_{2b}$ = 0.00680, $w_0$ = 0.14589 $w_1$ = 1, $w_2$ =6.36174 | BEB 6 > 0.5 |
| Model A null | 3 | $p_0$ = 0.59560, $p_1$ = 0.31827, $p_2$ = 0.05613, $p_{2b}$ = 0.02999, $w_0$ = 0.14518, $w_1$ = 1, $w_2$ = 1 | Not allowed |
| **Branch-specific: Swift** | | | |
| Model A | 4 | $p_0$ = 0.64711, $p_1$ = 0.34487 $p_{2a}$ = 0.00523, $p_{2b}$ = 0.00279, $w_0$ = 0.14656 $w_1$ = 1, $w_2$ = 19.70665 | BEB 4 > 0.5 |
| Model A null | 3 | $p_0$ = 0.62474, $p_1$ = 0.33425, $p_{2a}$ = 0.02672, $p_{2b}$ = 0.01429, $w_0$ = 0.14629, $w_1$ = 1, $w_2$ = 1 | Not allowed |
| **Branch-specific: Chicken** | | | |
| Model A ($\omega_i$=2) | 4 | $p_0$ = 0.64398, $p_1$ = 0.34260 $p_{2a}$ = 0.00876, $p_{2b}$ = 0.00466, $w_0$ = 0.14638, $w_1$ = 1, $w_2$ = 15.34900 | BEB 5 > 0.5 |
| Model A null | 3 | $p_0$ = 0.59240, $p_1$ = 0.31644, $p_{2a}$ = 0.05942, $p_{2b}$ = 0.03174, $w_0$ = 0.14625, $w_1$ = 1, $w_2$ = 1 | Not allowed |

## Likelihood Ratio Tests for T1R1:

| Comparison | Null Model lnL | Alt Model lnL | *df* | Adjusted deltaL | Critical Value | Significant? |
|---|---|---|---|---|---|---|
| ***Site Analysis*** | | | | | | |
| m0 v m3Discrtk2 | -43327.290829 | -42190.404734 | 2 | 2273.77219 | 5.99 | YES |
| m3Discrtk2 v m3Discrtk3($\omega_i$=1) | -42190.404734 | -42039.385219 | - | 302.03903 | 1 | YES |
| m1Neutral v m2Selection | -42448.130209 | -42448.130209 | 2 | 0 | 5.99 | NO |
| m7 v m8 | -42006.027666 | -41994.747585 | 2 | 22.560162 | 5.99 | YES |
| m8a v m8 | -41999.161582 | -41994.747585 | 1 | 8.827994 | 2.71 | YES |
| ***Branch Analysis*** | | | | | | |
| ***Hummingbird:*** | | | | | | |
| ModelA v m1Neutral | -42444.458400 | -42448.130209 | 2 | 7.343618 | 5.99 | YES |
| ModelA v ModelAnull | 42444.458400 | -42444.867420 | 1 | 0.81804 | 3.84 | NO |
| ***Swift:*** | | | | | | |
| ModelA v m1Neutral | -42445.464463 | -42448.130209 | 2 | 5.331498 | 5.99 | NO |
| ModelA v ModelAnull | -42445.464463 | -42447.454148 | 1 | 3.979376 | 3.84 | YES |
| ***Chicken:*** | | | | | | |
| ModelA ($\omega_i$=2) v m1Neutral | -42445.990310 | -42448.130209 | 2 | 4.279798 | 5.99 | NO |
| ModelA v ModelAnull | -42445.990310 | -42447.358941 | 1 | 2.737262 | 3.84 | NO |

$\omega_i$= initial starting value of $\omega$, if a better fit than ($\omega_i$=0), assessed via likelihood ratio tests

**Table S14: Description of sites in hummingbird T1Rs with evidence of positive selection.** Posterior probabilities and functional information related to positively selected codons identified in branch-site tests for a) T1R3 and b) T1R1.

**A) T1R3**

| Residue # | Amino acid | BEB posterior probability | Functional information regarding site |
|---|---|---|---|
| 206 | I | 0.694 | Important residue for the hummingbird sucrose response (this chimeric analysis) |
| 237 | S | 0.571 | Important residue for the hummingbird sucrose response (this chimeric analysis) |
| 372 | A | 0.593 | Aligns to S362 of hummingbird T1R1 (see Table S14B, below); 4 residues away from *Sac* phenotype site residue 371 |
| 384 | L | 0.641 | Aligns to human T1R2 residue 383, important for sucrose and sucralose response |
| 511 | Y | 0.804 | |
| 530 | Q | 0.985 | 1 residue away from cysteine-rich region important for brazzein response in human T1R3 |

**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**)** and 1 residue away from human T1R2 residue 65 (involved in sucralose response) |
| 58 | A | 0.858 | 3 residues away from human T1R1 residue 71 (important for ribonucelotide-potentiated umami response) |
| 362 | S | 0.702 | Aligns to A372 identified in hummingbird T1R3 analysis (Table S14A, above); 4 residues away from *Sac* phenotype site (residue 371) |
| 430 | G | 0.852 | |
| 449 | S | 0.520 | 1 residue away from human T1R1 residue 460, important for broad tuning of umami receptor |
| 580 | F | 0.773 | |