

## Research



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## Molecular evolution

# Functional divergence of bitter taste receptors in a nectar-feeding bird

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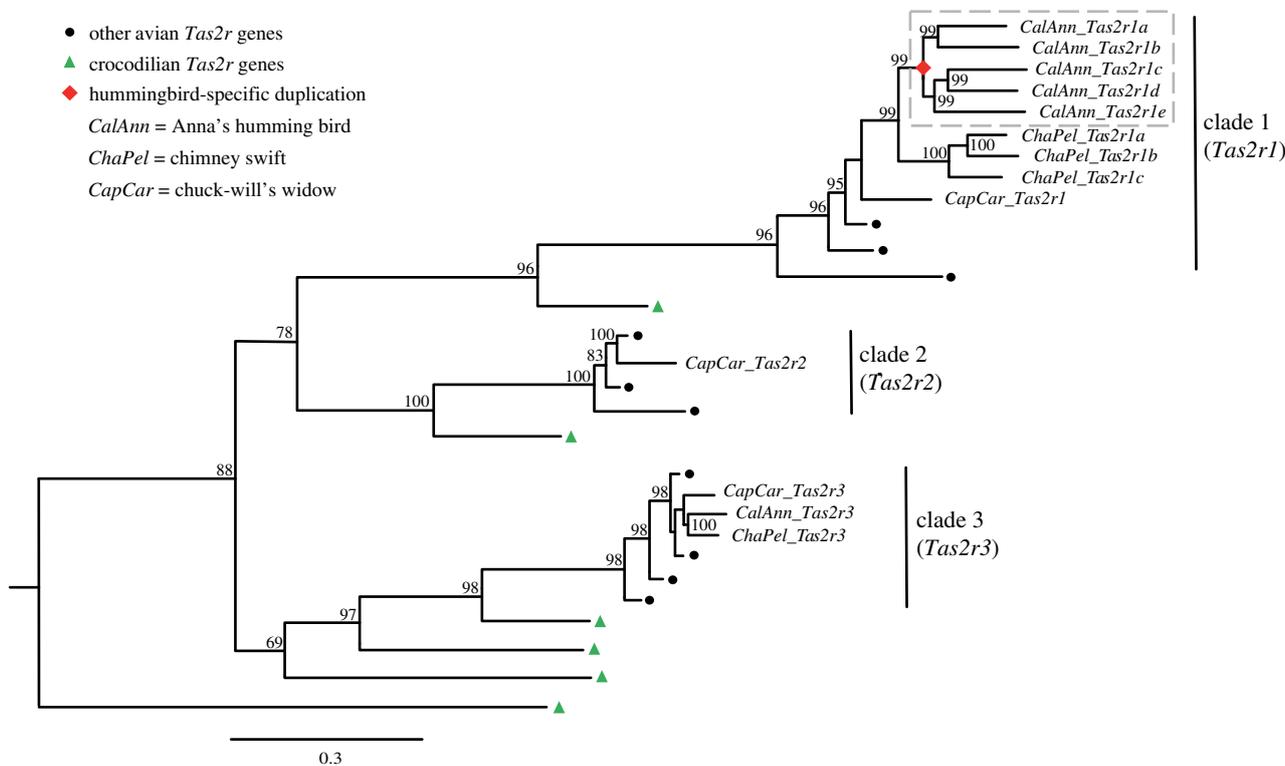
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Nectar may contain many secondary metabolites that are commonly toxic and bitter-tasting. It has been hypothesized that such bitter-tasting secondary metabolites might keep the nectar exclusive to only a few pollinators. To test this hypothesis, we examined functional changes of bitter taste receptor genes (*Tas2rs*) in a species of nectar-feeding bird (Anna's hummingbird) by comparing these genes with those from two closely related insect-feeding species (chimney swift and chuck-will's widow). We previously identified a larger number of *Tas2rs* in the hummingbird than in its close insectivorous relatives. In the present study, we demonstrate higher sensitivity and new functions in the hummingbird *Tas2r* gene copies generated by a lineage-specific duplication, which has been shaped by positive selection. These results suggest that the bitter taste may lead to increased sensitivities and specialized abilities of the hummingbird to detect bitter-tasting nectar. Moreover, this study potentially supports the hypothesis that bitter-tasting nectar may have been specialized for some pollinators, thus enforcing plant–pollinator mutualism.

## 1. Introduction

Bitterness is one of the five basic tastes in vertebrates, which typically functions as a natural defence to prevent the consumption of bitter-tasting toxins such as plant secondary metabolites and insect defensive secretions [1]. Bitter taste in vertebrates is mediated by a group of G protein-coupled receptors encoded by the type 2 taste receptor genes (*Tas2rs*) [2], the number of which ranges from 0 in the dolphin to 51 in the frog [3]. In general, the number of putatively functional *Tas2rs* in a vertebrate species is positively correlated with the abundance of potential toxins in its diet, supporting the hypothesis that dietary toxins have shaped the diversity of the *Tas2r* gene repertoire in vertebrates [3,4]. Although differences in feeding ecology could partially explain changes in *Tas2r* gene number in vertebrates, additional factors must be involved, because mismatches between feeding ecology and taste receptor evolution have also been identified [5–7].

Plant–pollinator mutualisms describe interactions whereby pollinators acquire food, and flowers achieve higher reproductive success through the spread of pollen by pollinators; such interactions are prevalent in nature [8]. Nectar is a sugar-rich liquid excreted from nectaries, and may contain many secondary metabolites that are commonly toxic and bitter-tasting, as a form of defence to some floral visitors [9,10]. Current techniques are available for the detection of very small traces of bitter-tasting compounds in nectar [11], and a number of studies have demonstrated that bitter-tasting metabolites are common in nectar, such as alkaloids, phenolics and non-protein amino acids [10]. One hypothesis to explain the purpose of such bad-tasting compounds is that these bitter-tasting secondary metabolites might keep the nectar exclusive to only a few pollinators [11,12]. To test this hypothesis, we examined functional



**Figure 1.** The simplified Bayesian gene tree of bird *Tas2rs*. Node support values represent Bayesian posterior probabilities shown as percentages, among which values lower than 50% were not shown. (Online version in colour.)

changes of bitter taste receptor genes (*Tas2rs*) in a species of nectar-feeding bird (Anna’s hummingbird, *Calypste anna*) and compared them to those of two closely related insect-feeding species (chimney swift, *Chaetura pelagica*, and chuck-will’s widow, *Caprimulgus carolinensis*) [13].

Following the release of avian genomes [13], we previously identified all complete and intact *Tas2r* sequences of 48 bird species [4]. A species-specific *Tas2r* gene duplication ( $n = 5$ ) was found to have occurred in the Anna’s hummingbird, which carries a larger *Tas2r* gene repertoire ( $n = 6$ ) compared with its two close relatives, the chimney swift ( $n = 4$ ) and the chuck-will’s widow ( $n = 3$ ). Both of these species are non-pollinators that mostly feed on insects [4]. In the present study, we conducted selective pressure analysis and a cell-based functional assay to test (i) whether *Tas2r* copies generated by the hummingbird-specific duplication have undergone positive selection and (ii) if these copies produce functional divergence.

## 2. Material and methods

### (a) Sequence data

All complete and intact *Tas2rs* from birds were taken from our previous work [4]. The simplified tree of avian *Tas2rs* (figure 1), which highlighted the evolutionary history of *Tas2rs* from the hummingbird and its two close relatives (chimney swift and chuck-will’s widow), was modified from figure 2 of our earlier publication [4]. For simplicity, we renamed all 13 *Tas2rs* from the three bird species studied here in order of appearance (figure 1).

### (b) Selective pressure analysis

To test whether the hummingbird-specific gene duplication has undergone positive selection, we estimated the ratio ( $\omega$ ) of non-synonymous to synonymous substitutions ( $d_N/d_S$ ) using the

Codeml program in PAML [14]. The improved branch-site model [15] was used to detect whether positive selection has affected additional sites along with the hummingbird-specific gene duplication. Thus, the foreground branches were set as all branches connecting the hummingbird (alternative model); the corresponding null model was the same as the alternative model, except  $\omega$  of the foreground branches was fixed at 1 [15,16]. The gene tree used for the selection test was taken from our previous study [4], but all genes from clades 2 and 3 (figure 1) were excluded, due to high sequence divergence between clades. For comparison, we additionally used two pairs of site-specific models in PAML to examine the five copies of hummingbird *Tas1rs* in clade 1 (figure 1). The first pair includes M1a and M2a; M1a assumes two classes of sites, one is constrained by  $0 < \omega < 1$  and the other is fixed at  $\omega = 1$ ; M2a is an extension of M1a, with an extra class of sites allowing  $\omega > 1$ . The second pair includes M8a and M8; M8a assumes a beta distribution for  $\omega$  among sites with  $0 < \omega < 1$ , but allows an extra class of sites to have  $\omega = 1$ ; M8 is same to M8a, but allows the extra class of sites to have  $\omega > 1$  [17]. Likelihood-ratio tests were used to identify significant evidence of positive selection by comparing two competing models [18].

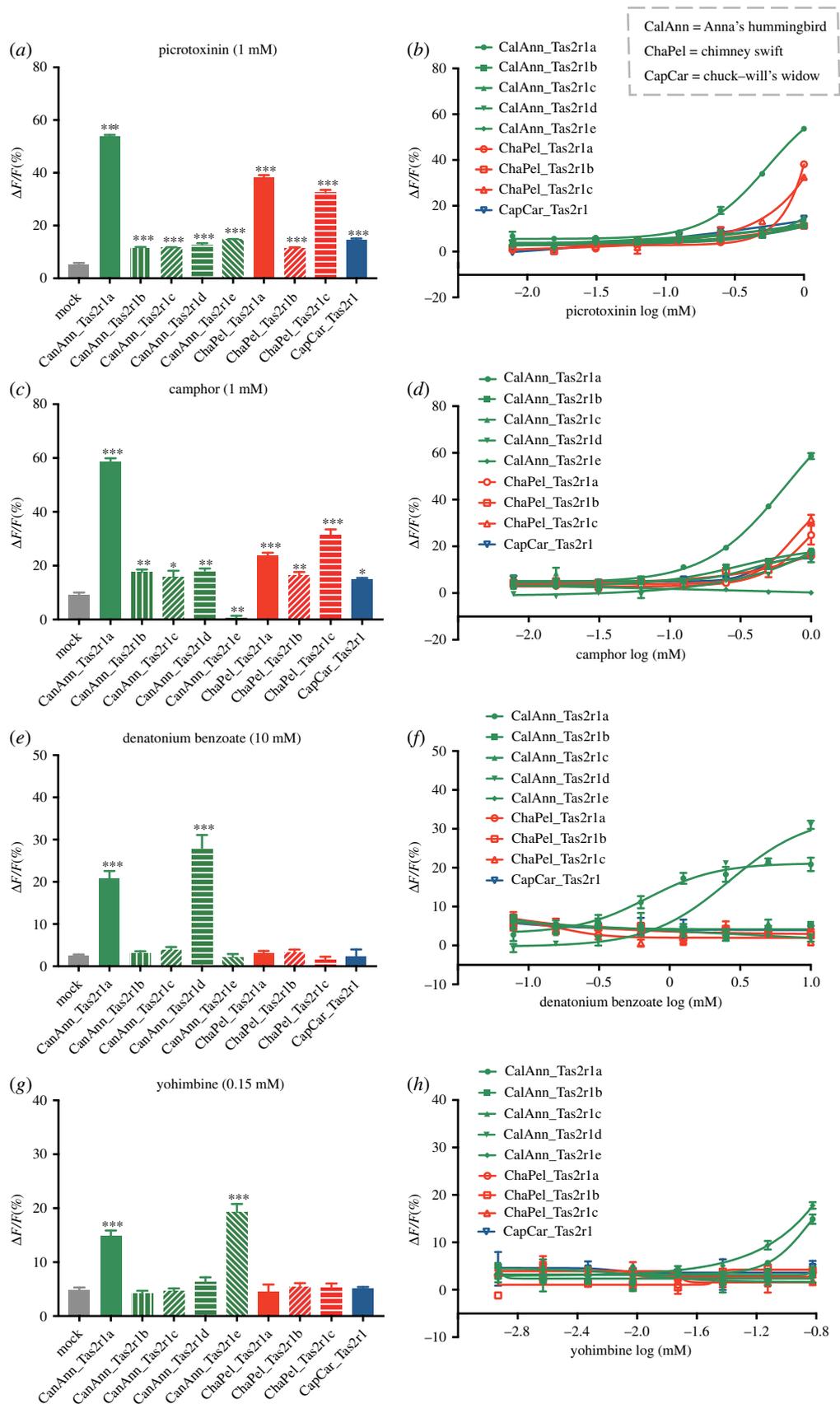
### (c) Functional assays

Information on the chemicals used in this study is presented in electronic supplementary material, table S1. Our cell-based functional assays were carried out as previously described [19,20], see also supplementary methods in the electronic supplementary material.

## 3. Results

### (a) *Tas2r* phylogeny and nomenclature

All avian *Tas2rs* formed three major clades in the gene tree (figure 1) [4]. For the three bird species studied here, clade 1



**Figure 2.** Functional divergence of Tas2r1 receptors in Anna's hummingbird and its two close relatives. HEK293 cells transiently transfected with an avian Tas2r1 receptor and a G $\alpha$ 16-gust44 were assayed for their responses to four compounds. (a,c,e,g) Quantitative analysis of responses of avian Tas2r1 receptors to picrotoxinin (1 mM), camphor (1 mM), denatonium benzoate (10 mM) and yohimbine (0.15 mM) (mean  $\pm$  s.e., \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , one-way ANOVA). (b,d,f,h) Dose-dependent responses of avian Tas2r1 receptors to picrotoxinin, camphor, denatonium benzoate and yohimbine. All data were fitted using GraphPad Prism 7.

contained the most genes: five in the hummingbird, three in the chimney swift and one in the chuck-will's widow. Clade 2 included only one gene (chuck-will's widow), and clade 3

consisted of three genes, one from each of the three species (figure 1). Gene duplication events were observed in clade 1 only: five copies in the hummingbird have been generated at

the origin of this species, while three copies have occurred at the origin of the chimney swift (figure 1). For convenience, we renamed genes in clade 1 as *Tas2r1*, those in clade 2 as *Tas2r2* and those in clade 3 as *Tas2r3*; multiple copies from one species in clade 1 were named alphabetically in order of appearance in the tree (figure 1). For clarity, the renamed gene sequences and their previous names are listed in electronic supplementary material, dataset S1.

### (b) Positive selection on duplicated *Tas2r* genes

To test whether *Tas2r1* duplicates specific to the hummingbird have undergone positive selection, we used the improved branch-site model [14]. We identified a small proportion of sites (5.7%) with a signature of positive selection ( $\omega = 6.226$ ,  $p = 1.55 \times 10^{-6}$ ; electronic supplementary material, table S2) in the dataset consisting of all 48 bird species from clade 1 (figure 1). Furthermore, seven positively selected sites were detected by this test (electronic supplementary material, table S2). In addition, the two site models M2a and M8 allowing positive selection are significantly different from M1a and M8a, respectively, suggesting that positive selection must have acted on the hummingbird *Tas2rs* (electronic supplementary material, table S3). M2a identified 10 positively selected sites, which are a subset of sites detected by M8 (electronic supplementary material, table S3). Notably, six sites that are putatively under positive selection were identified by both site models (M2a and M8) and the branch-site model (electronic supplementary material, tables S2 and S3), and four of them (91K, 92I, 181L and 271K) are located in extracellular loops that may mediate ligand recognition and receptor activation (electronic supplementary material, figure S1) [21].

### (c) Functional divergence of duplicated *Tas2r* genes

To determine whether duplication events of avian *Tas2rs* were accompanied by functional divergence, we conducted cell-based functional assays on all *Tas2r1* receptors in clade 1 (figure 1) from the hummingbird and its two close relatives. We expressed all nine *Tas2r1* receptors individually in HEK293 cells (PeakRapid) by transiently transfecting a *Tas2r1* construct along with a coupling chimeric G protein,  $G\alpha_{16}$ -gust44. We tested these *Tas2r1* receptors for their responses toward 24 commercially available bitter tasting compounds that have been shown to activate human *Tas2r* receptors (electronic supplementary material, table S1) [22]. Activation of each avian *Tas2r1* receptor was monitored by their relative fluorescence changes ( $\Delta F/F$ ). Mock-transfected cells were used as negative controls.

After screening all of the 24 bitter compounds (electronic supplementary material, table S1), we found that various *Tas2r1* receptors differed in the number of bitter compounds that elicited a response (electronic supplementary material, table S4). Specifically, some receptors seemed to be broadly tuned, such as CalAnn\_*Tas2r1a* and CapCar\_*Tas2r1*, which showed relatively wide-range responses toward six and five compounds, respectively. On the other hand, other receptors appeared to be activated by a narrow range of tuning (electronic supplementary material, figures S2 and S3). Based on the chemical screening results, we found that all nine of the *Tas2r1* receptors tested were able to recognize picrotoxinin, with different degrees of sensitivity; CalAnn\_*Tas2r1a* showed the highest level of response intensity (figure 2*a,b*). CalAnn\_*Tas2r1a* also appeared to be the most sensitive, followed by

ChaPel\_*Tas2r1a* and ChaPel\_*Tas2r1c*, while the others showed relatively low sensitivities (figure 2*b*). All of the receptors were also triggered by camphor, with the exception of CalAnn\_*Tas2r1e*, which showed no response at any concentrations tested. CalAnn\_*Tas2r1a* seemed to have the highest sensitivity to camphor, whereas the sensitivities of ChaPel\_*Tas2r1c* and ChaPel\_*Tas2r1a* are the second and third highest, respectively (figure 2*c,d*). Overall, functional differences were apparent among the five copies of Anna's hummingbird *Tas2r1*, and also between hummingbird *Tas2r1* copies and their outgroups, reflected by their different degrees of sensitivity in response to the examined bitter compounds.

Intriguingly, both CalAnn\_*Tas2r1a* and CalAnn\_*Tas2r1d* were responsive to denatonium benzoate, while other receptors showed no response at any concentrations tested (figure 2*e,f*). Similarly, yohimbine only elicited a response in the two receptors (CalAnn\_*Tas2r1a* and CalAnn\_*Tas2r1e*), but did not activate others (figure 2*g,h*). These results suggest that the gene duplication events in the hummingbird may have led to new gene functions, for example, the detection of denatonium benzoate and yohimbine. To test whether this potentially new function is also displayed in non-*Tas2r1* and non-hummingbird receptors, we additionally performed calcium mobilization assays for CalAnn\_*Tas2r3*, CapCar\_*Tas2r2*, CapCar\_*Tas2r3* and ChaPel\_*Tas2r3* (figure 1). Our results confirmed that none of these receptors were responsive to these two bitter compounds at any of the concentrations tested (electronic supplementary material, figure S4). Therefore, we conclude that the ability to detect denatonium benzoate and yohimbine is likely a novel function specific to the hummingbird *Tas2r1* receptors.

## 4. Discussion

Several hypotheses about the possible functions of toxic and bitter-tasting nectar have been proposed. One of which is the pollinator fidelity hypothesis, which assumes that specialized pollinators would be less repelled by toxic nectar than generalists [11,12]. In other words, this hypothesis argues that toxic nectar may maintain exclusivity to specific pollinators. In this study, we examined functional changes of bitter taste receptor genes (*Tas2rs*) in a nectar-feeding bird by comparing them with two closely related insect-feeding birds. Our results showed different levels of sensitivity and new functions in the hummingbird *Tas2r* gene copies, which may have been shaped by positive selection. These results suggest that the bitter taste may confer increased sensitivities and specialized abilities of the hummingbird to detect bitter-tasting nectar. In addition, this study potentially supports the hypothesis that toxic and bitter-tasting nectar may have played an important role in maintaining exclusivity to specific pollinators.

Birds generally have fewer *Tas2rs* compared to most other vertebrates [4]. In fact, some birds do not even possess any functional *Tas2rs* [23]. It has been hypothesized that herbivorous and insectivorous birds may require a higher number of *Tas2rs* than carnivorous birds that feed on non-insect animals, because plant products contain more toxins than animal tissues, and insects release toxic defensive secretions [4]. However, no significant differences have been found in the number of *Tas2rs* between nectarivorous and insectivorous birds [4]. Thus, it is surprising that the Anna's hummingbird—which mainly feeds on nectar—has a relatively higher

number of *Tas2rs* than its two close insectivorous relatives. This increased number was the result of a lineage-specific duplication, leading to the production of five *Tas2r* copies (figure 1) [4]. The five *Tas2r* copies showed varying sensitivities to picrotoxinin and camphor, as well as unique detection of denatonium benzoate and yohimbine, as demonstrated by our cell-based assays (figure 2). Intriguingly, *Tas2r* copies of the insectivorous chimney swift also showed relatively higher sensitivities to bitterness (figure 2*b,d*), which could be linked to the bitter-tasting defensive secretions in insects [24]. As a result, we cannot rule out the possibility that high sensitivities to bitterness are also needed in insectivorous birds. With the exception of denatonium benzoate, the three bitter compounds (picrotoxinin, camphor and yohimbine) are all naturally occurring chemicals extracted from plants. However, none of them are likely to exist in nectar other than camphor, which was isolated from the floral component [25]. Thus, future in-depth studies of bitter compounds in nectar distributed around hummingbird habitats would provide a better understanding of how hummingbirds use bitter taste to identify nectar in nature. Moreover, all the 24 bitter compounds tested in our assays have been verified to activate human *Tas2rs* [22], but only six, five and two are able to activate the hummingbird, chuck-will's widow, and chimney swift *Tas2rs*, respectively (figure 2 and electronic supplementary material, table S4). Thus, there is an apparent bias in bitter compounds that activate bird and human *Tas2rs*. Despite such limitations, the enhanced sensitivities to some bitter compounds and novel functions to detect new bitter compounds suggest that the hummingbird may use the bitter taste to detect nectar that have bitter-tasting secondary metabolites. Although it is long believed that the bitter taste is a natural defence against the ingestion of bitter-tasting and potentially toxic foods in the

wild [1], the functional enhancement and expansion of the hummingbird bitter receptors indicates that this nectar-feeding bird may prefer some bitter compounds compared to their insect-feeding relatives. This hypothesis is supported by a previous study, which showed that hummingbirds prefer nectar with a low-nicotine concentration [26]. Similarly, low-toxin nectar preference was also found in other pollinators such as honeybees [27]. As such, the bitter taste may be useful to help the hummingbird recognize specific and toxic nectar which non-pollinators may not be able to detect, thus enforcing plant–pollinator mutualism. It should be noted that our cell-based assays are *in vitro* tests, which cannot replace *in vivo* tests, although both types of tests remain a consistent trend on a qualitative level [28]. Moreover, the selection for bitterness in birds could be more complicated than the only relation with nectar. Regardless, our results should stimulate behavioural studies in the future to test whether nectar-feeding animals can prefer the bitter-tasting secondary metabolites in low concentrations from their nectar foods.

**Data accessibility.** *Tas2r* gene sequences used in our cell-based functional assays are provided in electronic supplementary material, dataset S1.

**Authors' contributions.** H.Z. conceived and designed the research and experiments; Y.W. performed the experiments; Y.W., H.J. and H.Z. analysed the data; P.J. supervised the cell-based functional assays; and Y.W. and H.Z. wrote the manuscript. All authors edited and approved the final version of the manuscript and agree to be held accountable for the content therein.

**Competing interests.** We declare we have no competing interests.

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