Evolvement of taste sensitivity and taste buds in chickens during selective breeding

Yuta Yoshida,[*,†,‡,1] Fuminori Kawabata,[§] Shoji Tabata,[‡] Samuel E. Aggrey,[#] Romdhane Rekaya,[||,†] and Hong-Xiang Liu[*,†,2]

[*]*Regenerative Bioscience Center, University of Georgia, Athens, GA, USA;* [†]*Department of Animal and Dairy Science, College of Agricultural and Environmental Sciences, University of Georgia, Athens, GA, USA;* [‡]*Laboratory of Functional Anatomy, Faculty of Agriculture, Kyushu University, Fukuoka, Japan;* [§]*Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Japan;* [#]*Department of Poultry Science, University of Georgia, Athens, GA, USA; and* [||]*Institute of Bioinformatics, University of Georgia, Athens, GA, USA*

ABSTRACT Chickens have been reported to have a low taste bud count and thus low taste acuity. However, more recent studies indicate that the earlier reported count of chicken taste buds may have been significantly underestimated. To answer the question of whether the taste sensing system in broiler chickens evolved during the breeding selection over the past decades, we compared the taste sensitivity to bitter and taste buds between a meat-type control strain − the 1955 Athens Canadian Random Bred (**ACRB**), and a modern high-yielding broiler strain − the 2012 Cobb 500. The behavioral tests showed that the ACRB did not avoid bitter taste solutions of quinine hydrochloride (**QHCl**) at the examined concentrations (0.5, 1, 2, and 4 mM) ($P > 0.05$), while the Cobb 500 significantly avoided both the 2 mM and 4 mM QHCl solutions ($P < 0.01$). The labeling of chicken taste buds using the molecular marker Vimentin revealed that Cobb 500 chickens had a slightly higher number ($P < 0.1$), but lower density of taste bud clusters in the palate ($P < 0.01$) and the base of the oral cavity ($P < 0.05$) compared to the ACRB. We also found that a single amino acid change occurred in the bitter taste receptor T2R7. However, the functional analyses using HEK293T cells transiently expressing T2R7 revealed that the functions of T2R7 were comparable between the two strains. Taken together, our results demonstrated that taste sensitivities could be affected by the selection of the broiler chickens. The modern high-yielding broilers, which have massive feed intake and appetite, had a higher sensitivity to bitter taste stimuli than the meat-type chicken strain which was established decades ago. This evolvement of taste sensitivities may be associated with the alterations of an upper level of taste system, rather than the peripheral taste system, including distribution of taste buds and functions of taste receptors.

Key words: Athens Canadian Random Bred, Cobb 500, bitter taste receptor, taste bud, taste sensitivity

INTRODUCTION

Taste buds are the peripheral sensory organs for taste that is essential in guiding animals to select and prioritize feed items and nutrients. Thus, taste buds play an important role in the feeding behavior of animals. Taste stimuli are received by taste receptors or channel proteins in the taste cells, which initiate the transduction of gustatory to neural signals (Roper and Chaudhari, 2017).

Chickens respond to bitter, umami, sour, salty, and high concentration of sweet taste stimuli (Urata et al., 1992; Cheled-Shoval et al., 2017; Yoshida et al., 2018a; Yoshida et al., 2018b; Yoshida et al., 2015). Among these taste stimuli, chickens are most sensitive to bitter tastants, including quinine hydrochloride (**QHCl**) as shown by Urata et al. (1992) and Cheled-Shoval et al. (2017). Bitter taste is mediated by the taste receptor type 2 (**T2R**) family (Mueller et al., 2005), and previous studies have identified three functional chicken T2Rs (T2R1, T2R2, T2R7) that are activated by various agonists ((Behrens et al., 2014; Hirose et al., 2015; Dey et al., 2017). A test based on QHCl solution intake (Hirose et al., 2015; Dey et al., 2017; Yoshida et al.,

2018a; Yoshida et al., 2018b) has been established to measure taste sensitivities in chickens. The test is brief and simple, and can be used for comparisons under different experimental conditions.

Chicken taste buds are mainly located in the palate and the base of the oral cavity (Ganchrow and Ganchrow, 1985; Kudo et al., 2008). It has been reported that chickens have a low number of taste buds and consequently a low taste acuity (Roura et al., 2013). Using immunoreactions against molecular markers to visualize chicken taste buds in the oral epithelial sheets, it has been demonstrated that chickens have more taste buds than previously reported (Rajapaksha et al., 2016; Venkatesan et al., 2016; Liu et al., 2018). In fact, it has been reported that modern broiler chickens have higher bitter taste sensitivity to QHCl than humans (Cheled-Shoval et al., 2017). Thus, it raises the question of whether taste sensitivity, the peripheral taste sensing system of broilers, evolved due to intensive selection for growth and feed intake.

The Athens Canadian Random Bred (**ACRB**), is a meat-type chicken control strain, was established in 1955 and has been maintained by the University of Georgia, Athens. It is the oldest known meat-type chicken control strain that still exists (Somes, 1988). The Cobb 500 (for hereafter Cobb 500) is a highly selected breed of broiler chickens. It is one of the most commonly raised high-yielding broiler strains achieving 3.7 to 4.7 times higher feed intake, and muscles production (breast and legs) when compared to the ACRB (Collins et al., 2014). In the current study, we compared taste sensitivities, taste bud number and density, and taste receptors between the ACRB and Cobb 500 in order to investigate the effects of over half century of intensive genetic selection on the taste sensing systems of broilers.

MATERIALS AND METHODS

Chemicals

Three bitter taste agonists in chickens, Quinine sulfate (**QS**; Wako Pure Chemical Industries, Osaka, Japan), dextramethorphan hydrochloride (**Dex**; Sigma-Aldrich, St. Louis, MO) and quinine hydrochloride (**QHCl**; Sigma-Aldrich, St. Louis, MO) were used as bitter stimuli (Behrens et al., 2014; Hirose et al., 2015; Dey et al., 2017). For the behavioral experiment, QHCl was dissolved in normal tap water just before each experiment. For Ca^{2+} imaging, these compounds were dissolved in ultra-pure water to make stock solutions, stored at $-20°$ C and diluted with a standard bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, adjusted pH value to 7.4 with NaOH just before each experiment.

Animals

The use of animals throughout the study was approved by The University of Georgia Institutional Animal Care and Use Committee and was in compliance with the National Institutes of Health Guidelines for the care and use of animals in research. Twenty (10 ACRB and 10 Cobb 500) 15-day old birds (**P15**) were used for 6 days to carry out the brief access test. For the immunohistochemistry (**IHC**) on the oral epithelial sheets and frozen sections, 10 ACRB and 20 Cobb 500 4-day-old birds (**P4**) were used. For nucleotide sequencing and molecular cloning of bitter taste receptors, 3 ACRB and 3 Cobb 500 20-day old birds (**P20**) were used. The chicks were euthanized by cervical dislocation at P4 for the IHC and at P20 for nucleotide sequencing and molecular cloning.

Brief Access Test

The brief access test was conducted largely as previously reported (Yoshida et al., 2018a; Yoshida et al., 2018b). Two cages (68.0 L × 68.8 W× 38.5 cm H) were used as experimental cage. A plastic box (8.3 L × 12.0 W × 5.1 cm H), filled with water or bitter solution, was placed at the center of each cage. In order to avoid solitary stress, a chick in one cage could see another chick in the neighboring cage, and we always used two chicks simultaneously, putting each of them separately into the two cages to measure their solution intakes. After the brief drinking session (5 min) in the experimental cage, the two chicks were moved back to the home cage. Then, the other two chicks were moved to the experimental cage for the next brief drinking session. This procedure was repeated for twenty birds (10 ACRB and 10 Cobb 500).

The chicks were restricted from water intake for a maximum of 24 h before each test to motivate drinking behavior. During experimental period, the body weight of birds didn't change (Figure 1B). The body weight gain might be affected by the water restriction. On day 1 and 2, each group of birds was provided access to water for 5 min in order to train them to drink water for a short time period. We provided additional water for 5 min after the training to minimize the variation of daily solution intakes among chicks. On day 3 to 6, the chicks were randomly allocated either into the water or QHCl solution intake groups (n = 5 in each group for both ACRB and Cobb 500 strains). The chicks allocated into the QHCl group were provided bitter solutions at QHCl concentrations of 0.5, 1, 2, and 4 mM. Birds in both groups were provided water or QHCl solutions for 5 min during days 3 to 6. Additional water was provided for 5 min to all chicks after each test.

Oral Epithelial Sheet Peeling

The oral epithelial sheets of chickens were prepared from the palate and the base of the oral cavity following reported methods (Rajapaksha et al., 2016). Briefly, an enzyme mixture of 1 mg/mL of Collagenase A (Roche Diagnostics, Rotkreuz, Switzerland) and 2.5 mg/mL of Dispase II (Roche Diagnostics) was injected into the subepithelial space of the palate and the base of the oral
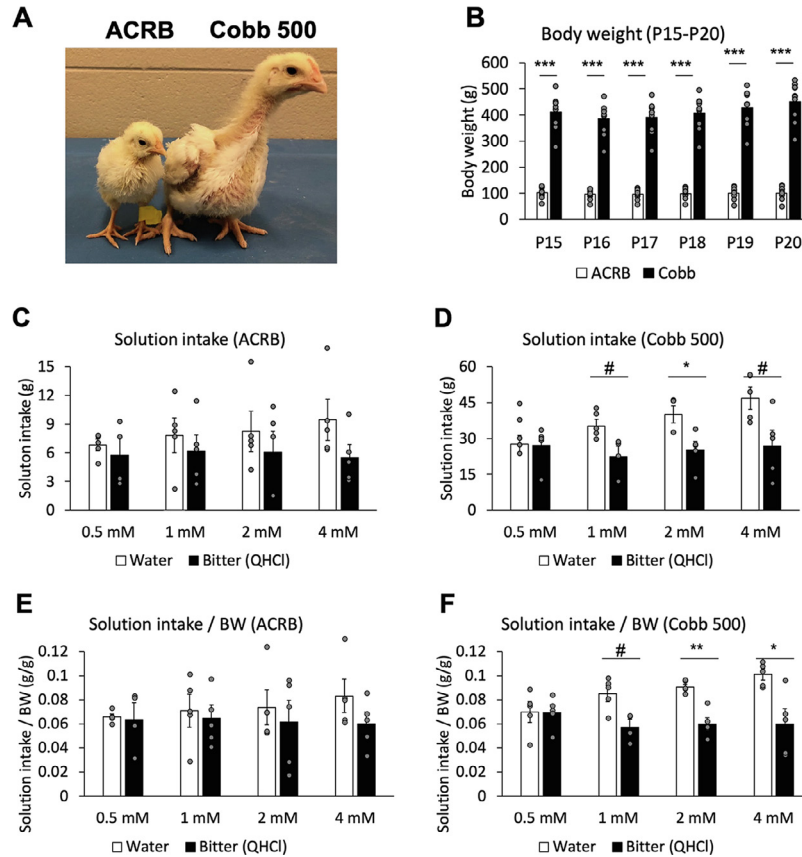
Figure 1. (A) The photograph of an Athens-Canadian Random Bred (ACRB) (left) and a Cobb 500 (right) chicken at P17. (B−F) Histograms (X±SE, n = 5−10) of the body weights (BW) of the ACRB and the Cobb 500 at P15-P20 (B), solution intakes (C, D) and solution intakes/BW (E, F) of water or bitter solutions containing 0.5, 1, 2, or 4 mM quinine hydrochloride (QHCl) of the ACRB (C, E) and the Cobb 500 (D, F). Dots in each bar represent the individual values of each animal. $^{\#}P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired t tests with Bonferroni correction.

cavity followed by incubation at 37°C for 90 min for the palate and 60 to 90 min for the base of the oral cavity. The tissues for immunoreactions were immediately fixed with 4% paraformaldehyde (**PFA**) for 60 min. Following a brief rinse in 0.1 M phosphate-buffered saline (**PBS**), the soft tissue regions containing taste buds were dissected from the beaks, and the epithelial sheets of the palate and the base of the oral cavity were peeled off from the underlying connective tissue. After thorough rinsing in PBS, the oral epithelial sheets were processed for immunohistochemistry.

Immunolabeling of Taste Buds in Oral Epithelial Sheets

The molecular marker Vimentin was used for labeling chicken taste buds in the oral epithelial sheets from the palate and the base of the oral cavity of chickens (Witt et al., 2000; Rajapaksha et al., 2016; Venkatesan et al., 2016). Non-specific staining was blocked by 10% normal donkey serum (**NDS**) in PBS with 0.3% Triton-X 100 (**PBS-X**) overnight at 4°C. The epithelial sheets were incubated with the mouse anti-Vimentin (1:500, V9, Thermo Fischer Scientific Inc., Waltham, MA) primary antibody in 1% NDS in PBS-X for 72 to 96 h at 4°C. After rinsing with PBS (3 times, 30 min each), the epithelial sheets were

incubated with Alexa Fluor 594 donkey anti-mouse secondary antibody (1:500, Jackson Immuno Research Laboratories Inc., ME) in 1% NDS in PBS-X overnight at 4°C. After rinsing in PBS (3 times 30 min each), the immunosignals were observed under an SZX2-ILLT Olympus stereomicroscope with CellSens software (Olympus, Life sciences, Tokyo).

Quantification of Number and Density of Taste Bud Clusters

Taste bud clusters labeled by immunosignals against Vimentin were quantified using photomicrographs of the oral epithelial sheets of chickens' palate and base of the oral cavity. The quantification was carried out by the same investigator for consistency among groups. The regions of the oral epithelial sheets where Vimentin^{+} taste buds were distributed were outlined, and the areas were measured using CellSens software (Olympus, Life sciences, Tokyo). The density of the taste bud clusters was calculated as the ratio between the number of taste bud clusters and area.

IHC on Frozen Sections

As the primary antibody, mouse anti-Vimentin (1:500) and rabbit anti-α-Gustducin (1:1000) was used

as chicken taste bud marker (Rajapaksha et al., 2016). To label the chicken bitter taste receptor, rabbit anti-T2R7 (1:1000), generated in our recent report (Yoshida et al., 2019) was used. As the secondary antibodies, Alexa Fluor 488 donkey anti-rabbit secondary antibody (1:500, Jackson Immuno Research Laboratories Inc., ME) and Alexa Fluor 594 donkey anti-mouse secondary antibody (1:500) were used.

The palate tissues were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan Co., LTd., Tokyo), and frozen at the $-80°C$. Sections were cut at 6 to 15 μm in thickness, and mounted onto gelatin-coated glass slides. Sections were fixed with 4% PFA for 10 min, and they were rehydrated in PBS. Nonspecific staining was blocked by 10% NDS in PBS-X for 30 min at room temperature. The sections were incubated with the primary antibodies in 1% NDS in PBS-X overnight at 4°C. The sections were rinsed with PBS (10 min) for 3 times. Then, the sections were incubated with the secondary antibodies in 1% NDS in PBS-X for 1 h at room temperature. The sections were rinsed with PBS (10 min) for 3 times again, and they were stained with DAPI (200 ng/mL in PBS) for 10 min, rinsed in PBS, air dried and coverslipped with ProLong® Diamond antifade mounting medium (P3697, Thermo Fisher Scientific Inc., MA). The fluorescent images were taken using a light microscope (EVOS FL, Light Technologies, CA).

Nucleotide Sequencing and Construction of Plasmids

Total RNA was isolated from the epithelium of the base of the oral cavity of 20-d old chicks, and first-strand cDNA was synthesized by reverse transcription. Deduced open reading frames (**ORFs**) of *T2R1, T2R2,* and *T2R7* were amplified and sequenced. The polymerase chain reaction (**PCR**) primers were designed based on the National Center for Biotechnology Information (**NCBI**) nucleotide databases of the *T2R1 (AB249766.1), T2R2 (AB249767.1),* and *T2R7* (NM_001080719.1). The entire sequences of *T2R1, T2R2,* and *T2R7* derived from ACRB and Cobb 500 chicks were compared with the genomic NCBI databases of *T2R1, T2R2,* and *T2R7.* The PCR products of the ORF of *T2R7* were subcloned into the pDisplay (Life Technologies Japan, Tokyo) mammalian expression vector by using an In-Fusion HD Cloning Kit (Takara Bio, Otsu, Japan). $G\alpha_{16/gust44}$/pcDNA3.1 (+) vector was kindly donated by Dr. Takashi Ueda (Nagoya City University).

Cell Culture

Human embryonic kidney (**HEK**)-derived 293T (**HEK293T**) cells were maintained in Dulbecco's Eagle's medium (DMEM high glucose; Wako) containing 10% fetal bovine serum (**FBS**; GE Healthcare, Buckinghamshire, UK), and penicillin-streptomycin solution (×100) (Wako) at 37°C and 5% CO_2.

Measurement of Cytosolic Ca^{2+} Concentrations

For the Ca^{2+} imaging experiments, HEK293T cells were transfected with either empty vector pDisplay for mock cells or co-transfection of $G\alpha_{16/gust44}$/pcDNA3.1 (+) with T2R7/pDisplay by using Screen$Fect^{TM}$A (Wako) on coverslips coated with poly-D-lysine (0.1 mg/mL; Wako). After transfection, the cells were incubated for 48 h at 37°C and 5% CO_2. The cells were then loaded with 1.25 μM Fluo 4-AM solution for 30 min at 37°C and 5% CO_2 in a dark room. Fluo 4-AM solution was prepared according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). The coverslips were washed with the standard bath solution, and Fluo 4 fluorescence was measured in the standard bath solution using a laser-scanning confocal microscope (Nikon A1R; Nikon Co., Tokyo). The coverslips were mounted in a chamber connected to a gravity flow system to deliver various stimuli. Chemical stimulation was applied by running a bath solution containing various chemical reagents. Cell viability was confirmed by the responses to 10 μM ATP.

Statistical Analysis

Statistical analyses were performed using Microsoft Excel (2011; Redmond, WA). Unpaired t-test with Bonferroni correction was used for testing. The Bonferroni correction was applied to account for multiple comparisons. A P-value <0.05 was used as threshold to declare statistical significance.

RESULTS

Cobb 500 Chickens Have a Higher Taste Sensitivity to Bitter Than ACRB

As expected, modern high-yielding broiler strain Cobb 500 chickens were significantly larger than the meat-type control strain ACRB (Figure 1A). Body weight (**BW**) of Cobb 500 birds was over 3-fold heavier their ACRB counterparts at P15 and P20 (P15-P20; $P < 0.001$). In fact, the average (SE) BW of Cobb 500 was 385.9 g (20.1 g) and 451.9 g (24.2 g) compared to 96.1 g (6.2 g) and 101.5 g (8.1g) for ACRB at P15 and P20, respectively (Figure 1B). In the brief access tests, solution intake by ACRB birds at 0.5, 1, 2, and 4 mM QHCl was not significantly different from water intake (Figure 1C) ($P > 0.05$). In contrast, solution intake by Cobb 500 birds decreased at 1, 2, and 4 mM of QHCl compared to water consumption (Figure 1D). The decrease was statistically significant QHCl concentrations of 2 mM or greater ($P < 0.05$). Comparison of solution intake standardized by BW showed similar trend. In fact, no significant difference was observed for the ACRB strain at all concentration levels (Figure 1E) ($P > 0.05$). For Cobb 500 birds, standardized solution intake decreased for QHCl concentration of 1 mM or

greater and was statistically significant for the highest two concentrations (Figure 1F) ($P < 0.01$).

Cobb 500 Chickens Have a Similar Number of Taste Bud Clusters as ACRB

The regions of the oral epithelial sheets that included taste buds were outlined and measured (Figure 2B, 3B). As a result, the gustatory areas of the oral epithelial sheets of the palate and the base of the oral cavity of the Cobb 500 were significantly larger than those of the ACRB (Figure 2C, 3C) (palate; $P < 0.001$, base of oral cavity; $P < 0.01$). Quantification of the numbers of taste bud clusters on the oral epithelial sheets of the palate (Figure 2D) and the base of the oral cavity (Figure 3D) showed a higher number of taste bud clusters in the palate of the Cobb 500 (275 ± 11) than that of the ACRB (number: 243 ± 13) (Figure 2D) ($P < 0.1$). However, the density of taste bud clusters (the numbers of taste bud clusters divided by the areas (mm^2)) on the oral epithelial sheets of the palate and the base of the oral cavity of the Cobb 500 was lower than that of the ACRB (Figure 2E, 3E) (palate; $P < 0.01$, base of oral cavity; $P < 0.05$). No apparent difference of taste bud number per cluster was observed between the two strains.

T2R7 is Mutated But Remains Functional During Genetic Improvement

Discrepancies of a single amino acid were detected in all three bitter taste receptors in both ACRB and Cobb 500 strains compared with the NCBI sequences. Of note, we found an amino acid mutation, Met94Leu, specific for the T2R7 of the ACRB (Figure 4). This amino acid position was predicted as an-agonist-interacting residue of chicken T2R7 (Di Pizio et al., 2018). Localization of T2R7 protein in the taste buds of the ACRB and Cobb 500 was confirmed by IHC on frozen sections (Figure 5).

Further, we performed functional analyses using HEK293T cells, transiently expressing either ACRB or Cobb 500 T2R7 with $G\alpha_{16/gust44}$. The response of the cells to 10 μM ATP was tested, and confirmed the viability for all cells (Figure 6A and 6B). Using the ratios of relative fluorescence units (**RFUs**), as an index of
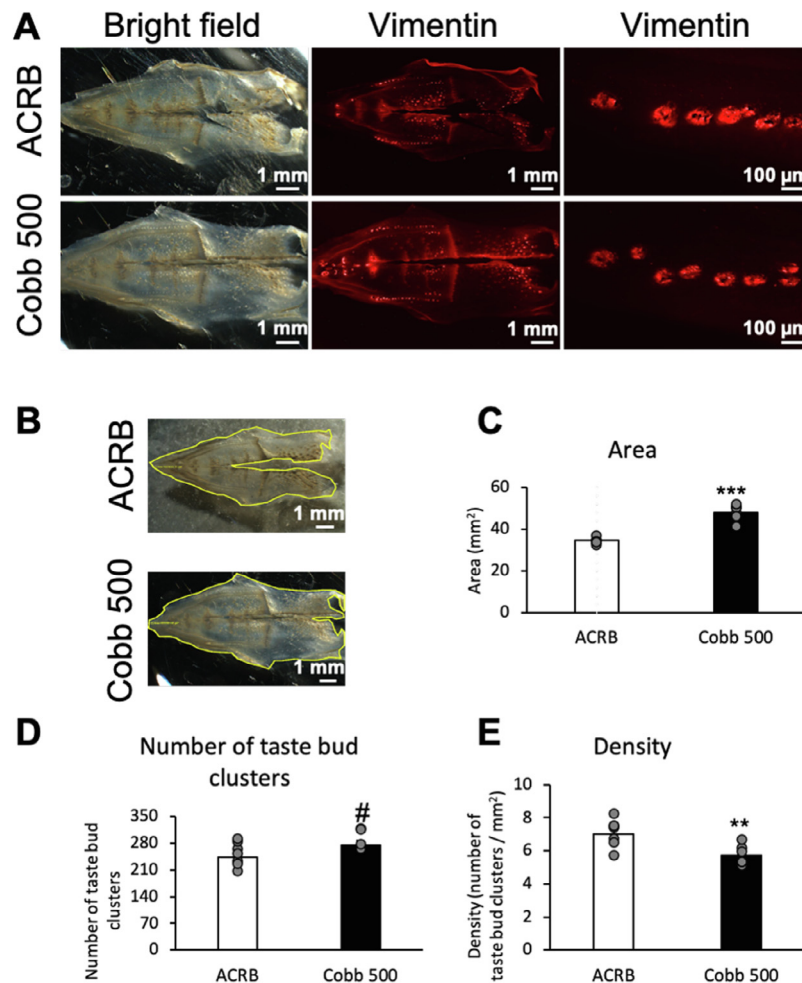

Figure 2. Quantification of taste bud number and density in the palate of the ACRB and Cobb 500 chickens at P4. (A) Photomicrographs of the epithelial sheets immunostained with a molecular marker for chicken taste buds, Vimentin (red). (B, C) Measurement (outlined in B) of the areas (mm^2) (C, X$\pm$SE, n = 6–7) of the palate epithelium that host taste buds. (D, E) Histograms to illustrate the numbers (D) and density (E) of taste bud clusters labeled with immunosignals of Vimentin on the epithelial sheets of the palate. The density of taste bud clusters were calculated as the number of taste bud clusters per mm^2. $^{\#}P < 0.1$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired t tests.
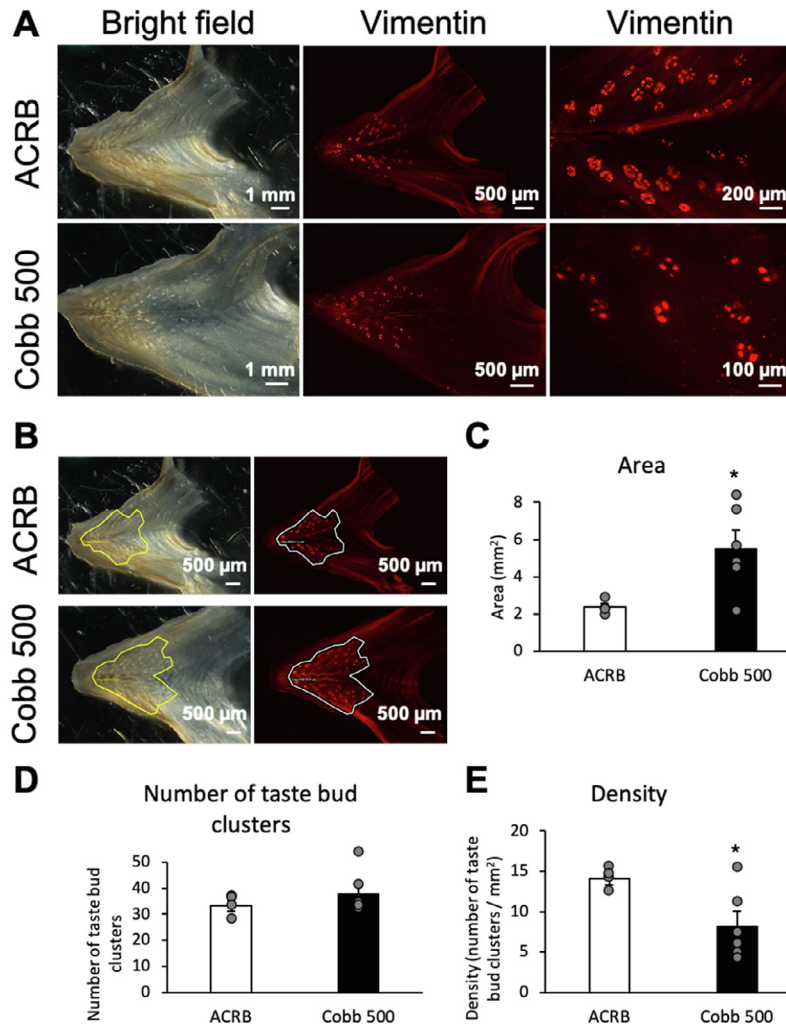
Figure 3. Quantification of taste bud number and density in the base of oral cavity in the ACRB and Cobb 500 chickens at P4. (A) The epithelial sheets (left) of the ACRB and the Cobb 500 of the base of the oral cavity were marked with the immunosignals of Vimentin (red) (middle and right). (B, C) The taste bud-containing areas (mm²) in the epithelial sheets of the base of the oral cavity were measured (outlines in B) and presented as a histogram (C, X±SE, n = 4−6). (D, E) Histograms of the numbers (D) and density (E) of taste bud clusters labeled with immunosignals of Vimentin on the epithelial sheets of the base of oral cavity. The density of taste bud clusters were calculated as the number of taste bud clusters per mm². *$P < 0.05$ by unpaired t tests.

cytosolic Ca^{2+} concentration, we found that HEK293T cells transiently expressing either Cobb 500 or ACRB T2R7 with $G\alpha_{16/gust44}$ exhibited similar RFUs in response to the stimulation of 100 μM quinine sulfate (**QS**) (A) or 100 μM dextromethorphan hydrochloride (**Dex**) (B) or 10 μM ATP to the base line value and to

T2R1			
Amino acid position	Gallus gallus	ACRB	Cobb 500
120	Ser	Ser, Ser/Thr, Thr	Ser/Thr, Ser/Thr, Thr
168	Ser	Ser, Ser/Tyr, Ser/Tyr	Ser, Ser/Tyr, Tyr
T2R2			
Amino acid position	Gallus gallus	ACRB	Cobb 500
296	Leu	Phe, Phe, Phe	Phe, Phe, Phe
T2R7			
Amino acid position	Gallus gallus	ACRB	Cobb 500
94	Met	**Leu, Leu, Leu/Met**	Met, Met, Met

Figure 4. Comparisons of amino acid sequences of the three chicken bitter taste receptors (T2R1, T2R2, and T2R7). Amino acids that are different from the chicken genome (*Gallus gallus*) obtained in the National Center for Biotechnology Information (NCBI) databases are shown. Individual data obtained from 3 ACRB chicks and 3 Cobb 500 chicks are listed.
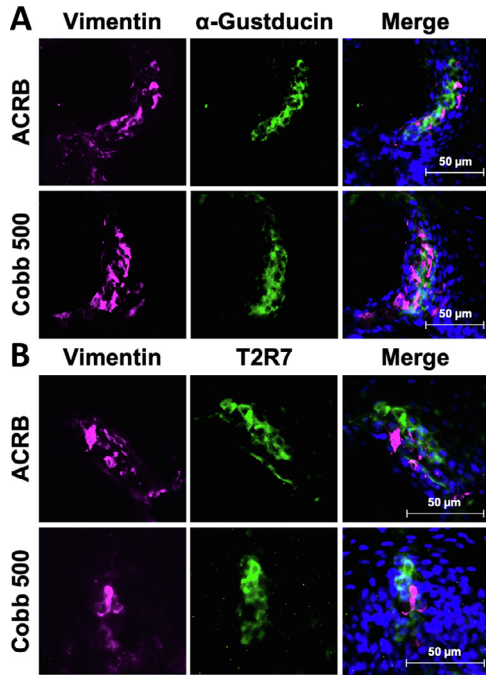
Figure 5. Localization of Vimentin (purple) and α-Gustducin (A) or T2R7 (B) (green) in the taste buds of the ACRB and Cobb 500. Merged images show the overlay of Vimentin (purple), α-Gustducin (A) or T2R7, and DAPI (blue) to stain nuclei.

the 10 μM ATP. The mock cells with an empty vector responded to 10 μM ATP, but not 100 μM quinine sulfate (**QS**) (Figure 6A) or 100 μM dextromethorphan hydrochloride (**Dex**) (Figure 6B). There were no apparent differences of maximum RFUs after stimulation by 30 to 300 μM QS (Figure 6C) or Dex (Figure 6D), normalized to those by 10 μM ATP in the HEK293T cells transiently expressing either Cobb 500 or ACRB T2R7 with $G\alpha_{16/\text{gust}44}$ ($P > 0.1$) (Figure 6C and 6D).

DISCUSSION

Chickens were historically believed to have a low taste bud count (conventional methods) and thus low taste acuity. However, many more taste buds were recently observed using immunohistochemical labeling against molecular markers in chicken taste buds (Rajapaksha et al., 2016). To understand the evolvement of taste sensing system due to intensive selection in broiler chickens, we analyzed the taste sensitivities, taste buds, and taste receptors in two very different strains of chickens: the ACRB, a control meat-type strain, and the Cobb 500, a modern high-yielding broiler strain. We found increased taste sensitivity to bitter in Cobb 500 chickens, however, the total number of taste bud clusters and taste receptor functions were comparable to the ACRB strain. As such, our results indicate an evolvement of taste sensitivity that is most likely not caused by the changes of taste buds and receptors.

Chickens are most sensitive to bitter stimuli even though they have a relatively small reservoir of bitter taste receptors, 3 subtypes (T1R1, T1R2 and T1R7) versus $\sim$25 in humans and $\sim$35 in mice (Shi & Zhang,

2005). It is noted that Cobb 500 are more sensitive to bitter stimuli than humans (Cheled-Shoval et al., 2017). Differences of bitter taste sensitivity between genders and among strains of chickens have also been reported. For example, the modern broiler strain Chunky, has a higher sensitivity to QHCl compared to the Rhode Island Red and the White Leghorn, both layer-type chickens (Kudo et al., 2010). Moreover, it has been found that taste sensitivities are heritable and related to the body weights of chickens (Barbato et al., 1982). From a chicken population divergently selected for growth, (Barbato et al., 1982) reported that the low BW line had higher bitter sensitivities compared to the high BW line using 24-week old adult chickens. In the current study, we used 15- to 20-day old chicks, and found that the Cobb 500 had higher bitter taste sensitivities compared to the ACRB. It is possible that bitter taste sensitivities can also be affected by growth stages, as reported previously (Dey et al., 2018). Previously, substantial researches using the ACRB strain have been conducted to understand the effects of selective breeding (Collins et al., 2016). Combining the present results with the previous report (Kudo et al., 2010), modern broiler strains, Cobb 500 and Chunky (ROSS), have higher bitter taste sensitivities, while layer-type strains, Rhode Island Red and White Leghorn, and control broiler strain, ACRB, have lower bitter taste sensitivities. Therefore, it is also plausible that the taste systems are co-selected with genetic improvement thereby leading to the alteration of taste sensitivities.

(Kudo et al. 2010) reported that the number of taste buds is related to bitter taste sensitivity. To understand whether the structure of peripheral taste tissues has evolved during the decades of intensive selection, we examined the numbers and density of taste bud clusters of ACRB and Cobb 500 strains. We found that the number of taste bud clusters in the palate and the base of oral cavity of Cobb 500 birds were only slightly higher than that of the ACRB. Due to the larger area of the oral cavities of Cobb 500 birds, their taste bud cluster density in the palate and the base of the oral cavity were lower than those of the ACRB. These data suggest that the difference in bitter sensitivities between Cobb 500 and ACRB is not caused by the change of density of taste bud clusters in the oral cavity.

It is considered that the expression levels or protein amounts of bitter taste receptors in the oral tissues could have been associated with the differences of bitter taste sensitivities between the ACRB and Cobb 500. In the present study, we have succeeded to amplify the ORFs of all bitter taste receptors from the oral tissues of the ACRB and Cobb 500. Therefore, we predict that certain amount of mRNAs of bitter taste receptors is present in the oral tissues of both strains. We also confirmed that the protein localization of T2R7 in the taste buds of the ACRB and Cobb 500 by the IHC on frozen sections (Figure 5). Thus, it is considered that the low bitter taste sensitivity of the ACRB strain is not derived from the lack of expression of bitter taste receptors in their taste buds.
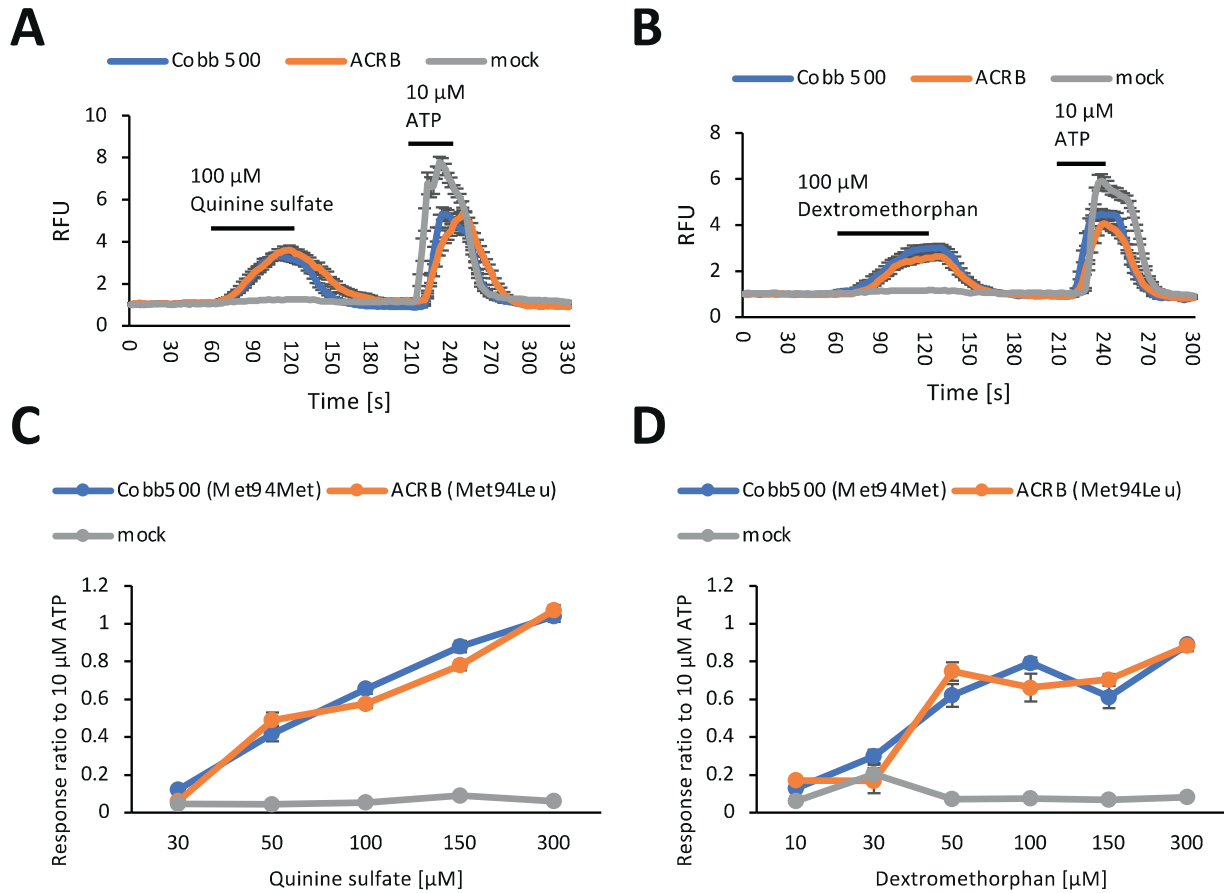
A

B

C

D

Figure 6. Responses of HEK293T cells transiently expressing the Cobb 500 T2R7 or ACRB T2R7 to bitter stimuli. (A-B) The representative data of the ratios of relative fluorescence units (RFUs), the index of cytosolic Ca^{2+} concentration, to the base line value after stimulation by 100 μM quinine sulfate (QS) (A) or 100 μM dextromethorphan hydrochloride (Dex) (B), or 10 μM ATP in the HEK293T cells transiently expressing either Cobb 500 T2R7 with $G\alpha_{16/gust44}$, or ACRB T2R7 with $G\alpha_{16/gust44}$, or empty vector (mock). (C-D) The maximum RFUs after stimulation by 30-300 μM QS (C) or Dex (D), normalized to those by 10 μM ATP in the HEK293T cells transiently expressing either of Cobb 500 T2R7 with $G\alpha_{16/gust44}$, ACRB T2R7 with $G\alpha_{16/gust44}$, and empty vector (mock). Values are the means $\pm$ SE (n $=60-90$ cells from 3 coverslips for each concentration). There were no statistically significant differences in responses to bitter stimuli between the Cobb 500 T2R7 and ACRB T2R7 by unpaired t tests.

We then asked whether mutations occurred in the bitter taste-related genes resulting in changing the function of bitter taste sensing or transduction leading to a change in bitter sensitivity. Analyses of nucleotide sequences of all three chicken bitter taste receptors, *T2R1, T2R2,* and *T2R7,* detected a specific amino acid change at the T2R7 (Met94Leu) in the ACRB. Previous *in silico* study predicted that this amino acid position was one of the agonist-interaction sites of chicken T2R7 (Di Pizio et al., 2018). However, the functional analyses of chicken T2R7 revealed no difference in the responses to 30 to 300 μM quinine sulfate (**QS**) and dextramethorphan hydrochloride (**Dex**) between the strains. Di Pizio et al. (2018) showed that multiple agonist-interaction sites in chicken T2R7 are responsible for the agonist recognition. Thus, it is possible that a single mutation of Met94Leu is not sufficient to change the T2R7 function.

Taken together, we found that modern high-yielding broiler Cobb 500 had higher bitter taste sensitivities, compared to the control strain ACRB, suggesting co-selection of taste behavior. Analyses of the distribution of taste buds, nucleotide sequencing, and functional analyses of T2Rs revealed that it is not the alterations in the peripheral taste system that caused this evolution of behavioral sensitivities between these two strains.

DISCLOSURES

The authors declare no conflict of interest.

REFERENCES

Barbato, G. F., P. B. Siegel, and J. A. Cherry. 1982. Genetic analyses of gustation in the fowl. Physiol. Behav. 29:29–33.

Behrens, M., S. I. Korsching, and W. Meyerhof. 2014. Tuning properties of avian and frog bitter taste receptors dynamically fit gene repertoire sizes. Mol. Biol. Evol. 31:3216–3227.

Cheled-Shoval, S. L., N. Reicher, M. Y. Niv, and Z. Uni. 2017. Detecting thresholds for bitter, umami, and sweet tastants in broiler chicken using a 2-choice test method. Poult. Sci. 96:2206–2218.

Collins, K. E., B. H. Kiepper, C. W. Ritz, B. L. McLendon, and J. L. Wilson. 2014. Growth, livability, feed consumption, and carcass composition of the Athens Canadian Random Bred 1955 meat-type chicken versus the 2012 high-yielding Cobb 500 broiler. Poult. Sci. 93:2953–2962.

Collins, K. E., H. L. Marks, S. E. Aggrey, M. P. Lacy, and J. L. Wilson. 2016. History of the Athens Canadian random bred and the Athens random bred control populations. Poult. Sci. 95:997–1004.

Dey, B., F. Kawabata, Y. Kawabata, Y. Yoshida, S. Nishimura, and S. Tabata. 2017. Identification of functional bitter taste receptors and their antagonist in chickens. Biochem. Biophys. Res. Commun. 482:693–699.

Dey, B., F. Kawabata, Y. Kawabata, S. Nishimura, and S. Tabata. 2018. Bitter taste sensitivity and the expression of bitter taste receptors at different growth stages of chicks. J. Poult. Sci. 55:204–209.

Di Pizio, A., N. Shy, M. Behrens, W. Meyerhof, and M. Y. Niv. 2018. Molecular features underlying selectivity in chicken bitter taste receptors. Front. Mol. Biosci. 5:6.

Ganchrow, D., and J. R. Ganchrow. 1985. Number and distribution of taste buds in the oral cavity of hatchling chicks. Physiol. Behav. 34:889–894.

Hirose, N., Y. Kawabata, F. Kawabata, S. Nishimura, and S. Tabata. 2015. Bitter taste receptor T2R1 activities were compatible with behavioral sensitivity to bitterness in chickens. Biochem. Biophys. Res. Commun. 460:464–468.

Kudo, K., S. Nishimura, and S. Tabata. 2008. Distribution of taste buds in layer-type chickens: scanning electron microscopic observations. Anim. Sci. J. 79:680–685.

Kudo, K. I., J. I. Shiraishi, S. Nishimura, T. Bungo, and S. Tabata. 2010. The number of taste buds is related to bitter taste sensitivity in layer and broiler chickens. Anim. Sci. J. 81:240–244.

Liu, H. X., P. Rajapaksha, Z. Wang, N. E. Kramer, and B. J. Marshall. 2018. An update on the sense of taste in chickens: a better developed system than previously appreciated. J. Nutr. Food Sci. 8:686.

Mueller, K. L., M. A. Hoon, I. Erlenbach, J. Chandrashekar, C. S. Zuker, and N. J. Ryba. 2005. The receptors and coding logic for bitter taste. Nature 434:225–229.

Rajapaksha, P., Z. Wang, N. Venkatesan, K. F. Tehrani, J. Payne, R. L. Swetenburg, F. Kawabata, S. Tabata, L. J. Mortensen, S. L. Stice, R. Beckstead, and H. X. Liu. 2016. Labeling and analysis of chicken taste buds using molecular markers in oral epithelial sheets. Sci. Rep. 6:37247.

Roper, S. D., and N. Chaudhari. 2017. Taste buds: cells, signals and synapses. Nat. Rev. Neurosci. 18:485–497.

Roura, E., M. W. Baldwin, and K. C. Klasing. 2013. The avian taste system: potential implications in poultry nutrition. Anim. Feed Sci. Technol. 180:1–9.

Shi, P., and J. Zhang. 2005. Contrasting modes of evolution between vertebrate sweet/umami receptor genes and bitter receptor genes. Mol. Biol. Evol. 23:292–300.

Somes, R. G. 1988. International Registry of Poultry Genetic Stocks. The University of Conneticut, Storrs, CT.

Urata, K., M. Manda, and S. Watanabe. 1992. Behavioral study on taste responses of hens and female Japanese quail to salty, sour, sweet, bitter and umami solutions. Anim. Sci. Technol. 63:325–331.

Venkatesan, N., P. Rajapaksha, J. Payne, F. Goodfellow, Z. Wang, F. Kawabata, S. Tabata, S. Stice, R. Beckstead, and H. X. Liu. 2016. Distribution of alpha-Gustducin and Vimentin in premature and mature taste buds in chickens. Biochem. Biophys. Res. Commun. 479:305–311.

Witt, M., K. Reutter, D. Ganchrow, and J. R. Ganchrow. 2000. Fingerprinting taste buds: intermediate filaments and their implication for taste bud formation. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355:1233–1237.

Yoshida, Y., Y. Kawabata, F. Kawabata, S. Nishimura, and S. Tabata. 2015. Expressions of multiple umami taste receptors in oral and gastrointestinal tissues, and umami taste synergism in chickens. Biochem. Biophys. Res. Commun. 466:346–349.

Yoshida, Y., F. Kawabata, Y. Kawabata, S. Nishimura, and S. Tabata. 2018a. Short-term perception of and conditioned taste aversion to umami taste, and oral expression patterns of umami taste receptors in chickens. Physiol. Behav. 191:29–36.

Yoshida, Y., F. Kawabata, Y. Kawabata, S. Nishimura, and S. Tabata. 2018b. Expression levels of taste-related genes in palate and tongue tip, and involvement of transient receptor potential subfamily M member 5 (TRPM 5) in taste sense in chickens. Anim. Sci. J. 89:441–447.

Yoshida, Y., Z. Wang, K. F. Tehrani, E. G. Pendleton, R. Tanaka, L. J. Mortensen, S. Nishimura, S. Tabata, H. X. Liu, and F. Kawabata. 2019. Bitter taste receptor T2R7 and umami taste receptor subunit T1R1 are expressed highly in Vimentin-negative taste bud cells of chickens. Biochem. Biophys. Res. Commun. 511:280–286.