Bitter Taste Sensitivity and the Expression of Bitter Taste Receptors at Different Growth Stages of Chicks

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Bitterness is one of the five basic tastes, and sensitivity to bitterness is important in that it enables animals to avoid harmful and toxic substances. In humans, taste sensitivity decreases with age, although the extent of loss varies depending on the taste quality. In chickens (*Gallus gallus domesticus*), baby chicks have been found to be more sensitive to salt and sour taste qualities than adults. In this study, therefore, we investigated the growth-associated changes in bitter taste sensitivity in chicks. We examined the behavioral perceptions toward the bitter compounds chloramphenicol and andrographolide in chicks at three different growth stages. Then, we measured the relative expression of the functional bitter taste receptors in the chick palate. In behavioral drinking tests, the 0-1-week-old chicks consumed a significantly lower amount of bitter solutions than water, whereas the 8-9-week-old chicks showed lower avoidance of the bitter solutions than the 0-1-week-old and 4-5-week-old chicks. Real-time PCR assay showed that the 0-1-week-old chicks had significantly higher expression of one of the functional bitter taste receptors in the palate than that in the older chicks. These results suggest that baby chicks are more sensitive to bitterness than older chicks. These findings may be useful in the production of new feedstuff for chicks according to their growth stages.

Key words: bitterness, chicken, growth, taste sense

Introduction

The sense of taste is important for evaluating the quality of nutrients and distinguishing between safe and dangerous foods prior to ingestion (Yarmolinsky *et al*., 2009; Chaudhari and Roper, 2010). Bitterness is one of the five basic tastes, and bitter taste perception in vertebrates is important in that it enables them to avoid harmful and toxic substances. The behavior of animals, especially their feeding behavior, is affected by taste receptor type 2 (T2R) members, which can induce them to avoid generally bitter, toxic, and harmful substances (Go *et al*., 2005). Recent studies of chicken (*Gallus gallus domesticus*) taste systems, using the chicken taste bud markers vimentin and α-gustducin (Rajapaksha *et al*., 2016; Venkatesan *et al*., 2016), have revealed the pre-

sence of many taste buds in the oral cavity and that chickens can detect some taste qualities, such as fat, bitter, and umami, *via* these taste receptors (Kudo *et al*., 2014; Hirose *et al*., 2015; Sawamura *et al*., 2015; Yoshida *et al*., 2015; Yoshida *et al*., 2018; Kawabata *et al*., 2018).

In humans, taste sensitivity decreases with age, although the extent of the loss varies depending on taste quality (Mojet *et al*., 2003). In chickens, very little research has been done to investigate growth-related taste loss and its subsequent effect on the animal's production. Using behavioral experiments, baby chicks were found to be more sensitive to salt and sour taste qualities than the adults were (Berkhoudt, 1985). However, to our knowledge, no previous studies have specifically explored the growth-associated effect on bitter taste perception in chicks. It is important to elucidate the bitter taste sensitivity at different growth stages of chicks because of the high variation in nutrient requirements during these developmental stages. The commercial feed industry produces different categories of chick feed, such as starter, grower, and finisher, on the basis of the growth stages of chicks.

Although chickens have three bitter taste receptor genes, chicken *T2R1* (*cT2R1*), *T2R2* (*cT2R2*), and *T2R7* (*cT2R7*) (Go, 2006), we confirmed through use of *in vitro* and *in vivo* techniques that only two of these receptors (cT2R1 and

cT2R7) are functional in the oral epithelium (Dey *et al.*, 2017). Gustducin is a taste-cell-specific G protein and an important mediator of bitter, sweet, and umami signal transduction (McLaughlin *et al.*, 1992; Wong *et al.*, 1996; He *et al.*, 2004). It is also specifically expressed in chicken taste cells (Kudo *et al.*, 2010). Therefore, in this study, we investigated the growth-associated changes in bitter taste perception in chicks by analyzing the expression changes of the functional bitter taste receptors and the gustducin alpha-subunit (*α*-gustducin) with growth.

From the results, we confirmed that younger chicks have a higher aversion to bitter substances than do older chicks, and also that 0−1-week-old chicks have the highest expression of one of the functional bitter taste receptors.

Materials and Methods

Chemicals

Chloramphenicol and andrographolide were purchased from Wako Pure Chemical Industries (Osaka, Japan) for the behavioral experiments. These compounds were dissolved in normal tap water just before each experiment to make bitter solutions and were then maintained at room temperature.

Animals

Rhode Island Red chicks of 0−1 week, 4−5 weeks, and 8−9 weeks of age and in good health were used for the behavioral experiments and palatine tissue sampling. We used male and female chicks randomly. The study was carried out according to the Guide for Animal Experiments issued by Kyushu University, the Law Concerning the Human Care and Control of Animals (Law No. 105; October 1, 1973), the Act on the Welfare and Management of Animals (Law No. 105; 1973, the Ministry of Environment), and the Japanese Government Notification on the Feeding and Safekeeping of Animals (Notification No. 6; March 27, 1980). The present animal experiments were approved by Kyushu University's Animal Experiments Review Board (Approval number: A28-151-1).

Behavioral Test

One-bowl drinking tests were conducted according to our previous report (Dey *et al.*, 2017). In brief, the behavioral test took place over 12 consecutive days, where the first six days were considered to be a training period for the chicks,

and days 7−12 were considered the experimental period. Commercial layer feed (metabolizable energy $>$ 2800 kcal/kg; crude protein $>$ 16.0%) was fed to the chicks on an *ad libitum* basis throughout the whole experiment (Powerlayer 17Y; JA Kitakyushu Kumiai Shiryo, Fukuoka, Japan). On the first day, the chicks were supplied normal tap water for 24 h and then the drinking time was restricted to only 10 min in each 24-h period beginning at 17:00, to train them in drinking for a short period of time. Over the experimental period (days 7−12), the chicks were supplied either water or a bitter solution (chloramphenicol or andrographolide) for 10 min. The water and bitter solutions were given on a randomized basis over the experimental period. To compensate for evaporation loss from the bowl over the 10 min of exposure, control tap water was set in a brooder box, and the amount of evaporation was subtracted from the volume of water or bitter solution intake. In the present behavioral tests, we used three different concentrations of chloramphenicol and androgrphoilde to prepare bitter solutions, where the highest compound concentrations were based on a previous cell-based assay report (Behrens *et al.*, 2014).

Real-Time PCR

First, total RNA was extracted from the palatine sample (tissue samples of approximately 100−200 mg) using ISOGEN II (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. For the elimination of genomic DNA, the extracted total RNA samples were subjected to DNase treatment using DNase I (Nippon Gene Co., Ltd.) according to the manufacturer's instructions.

Next, real-time PCR was performed on an Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA, USA) using the One Step SYBR PrimeScript RT-PCR Kit II (Perfect Real Time) (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Gene-specific primers for *cT2R1*, *cT2R7*, *α-gustducin*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were generated according to their known cDNA sequences (Table 1). All primers were designed for an annealing temperature of 57℃−60℃ and were compatible for forward and reverse primers (which were up to 3℃ apart). The PCR mixture had a total volume of 10 μl and consisted of 2.8 μl of RNase-free water, 5.0 μl of 2× One Step SYBR RT-PCR Buffer 4 (TaKaRa Bio Inc.), 0.3 μl

Table 1.　**Primers used for real-time PCR**

Target genes	Accession no.		Primer sequence (5′ -3′)	Product size (bp)
cT2R1	AB249766.1	Forward	CCAGTCATTCCTCAGCTGGC	131
		Reverse	AGTTGCTGTGTGCGTTGTAG	
cT2R7	NM_001080719	Forward	CCAGTCATTCCTCAGCTGGC	138
		Reverse	GTAGTGAAGTTGCTGTGTGCG	
α-Gustducin	NM_001267811.1	Forward	TGCACTCCCGAGTGAAAACAA	100
		Reverse	CTCTCTGATCGCTGCCACC	
GAPDH	NM_204305.1	Forward	ACTGTCAAGGCTGAGAACGG	99
		Reverse	ACCTGCATCTGCCCATTTGA	

of PCR primer forward (10 μM), 0.3 μl of PCR primer reverse (10 μM), 0.2 μl of ROX Reference Dye II (TaKaRa Bio Inc.), 0.4 μl of PrimeScript One Step Enzyme Mix 2 (TaKaRa Bio Inc.), and 1.0 μl of total RNA (100 ng/μl). The PCRs were conducted under the following conditions: 42℃ for 5 min, 95℃ for 10 s, and 40 cycles of 95℃ for 5 s and 60℃ for 34 s. In addition, to verify the amplification of a single product, a stage with a temperature increment to generate a melting curve was conducted under the following conditions: 95℃ for 15 s and 60℃ for 1 min, followed by a temperature increment of 95℃ for 15 s. The fluorescent SYBR Green signal was measured immediately after the extension step of each cycle, and the cycle in which the product was first detectable was recorded as the cycle threshold. To control for false positives, a non-template control was run for each template and primer pair.

Statistical Analysis

The data are expressed as the means±SE. Statistical analyses were performed using the paired t-test or Tukey's test. The analyses were performed with the IGOR Pro software package (Version 6.34J; WaveMetrics, Portland, OR, USA), and differences with P-values ＜0.05 were considered significant.

Results

Behavioral Sensitivity of Younger and Older Chicks

The 0−1-week-old chicks consumed significantly lower amounts of 1.0 and 0.33 mM chloramphenicol solutions than water, whereas the 4−5-week-old chicks consumed significantly lower amounts of 1.0 mM chloramphenicol solution only (Fig. 1). Interestingly, the 8−9-week-old chicks did not show any aversive behavior to any of the chloramphenicol solutions tested (Fig. 1).

In another set of experiments, the 0−1-week-old chicks showed similar aversions to 0.3 and 0.1 mM andrographolide solutions compared with water in a 10-min drinking test, but the 4−5-week-old and 8−9-week-old chicks did not show any aversion to any of the doses tested here (Fig. 1).

Relative Expression of cT2R1 and cT2R7 mRNAs in the Chick Palate

Real-time PCR was used to determine the mRNA expression of bitter taste receptor genes and their signal transduction protein transcripts in the palate tissue. For *cT2R1*, the 0−1-week-old chicks had significantly higher mRNA expression than both the 4−5-week-old and 8−9-week-old chicks had when normalized to *GAPDH* mRNA (Fig. 2A), but they had higher mRNA expression than that of the 8−9-week-old chicks only when normalized to the *α-gustducin* mRNA (Fig. 2B).

Additionally, the relative expression levels of *cT2R7* in the palate were not significantly different among the three growth stages of chicks for both normalization methods (Figs. 2C, D). Furthermore, we confirmed that the growth stage of chicks did not affect the expression of *α-gustducin* and *GAPDH* mRNAs (Figs. 2E, F).

Discussion

In this study, we found that older chicks had a significantly higher bitter taste threshold than that of the younger chicks. We also found that the mRNA expression level of one of the functional palatine bitter taste receptors, *cT2R1*, was significantly lower in the older chicks than in the younger chicks, although the *cT2R7* expression levels were not statistically different. These results imply that the reduction in bitter taste sensitivity in chicks during growth is induced by a decrease in the expression of the functional bitter taste receptor in the oral tissues. We have previously reported that cT2R1 activities were compatible with behavioral sensitivity to bitterness (Hirose *et al*., 2015). Taken together, these findings suggest that both the activities and expression levels of cT2R1 determine the intensity of the taste of bitterness in chicks.

Chloramphenicol is a promiscuous ligand for cT2Rs, whereas andrographolide is selective for cT2R7 only (Behrens *et al*., 2014). Although the *cT2R7* expression levels were not statistically different, they were slightly decreased with growth. Since chicks showed a reduction of bitter taste sensitivity for both compounds with growth, it is possible that cT2R7 expression may decrease with growth at the protein level. Further studies are needed to confirm this.

We measured the relative expression levels of *cT2R1* and *cT2R7* in the palate and normalized them to the levels of both *α-gustducin* and other internal standard molecules. The normalization with *α-gustducin* yields the expression level per taste cell, because *α*-gustducin is a specific marker of a part of the taste cell in chickens (Kudo *et al*., 2010). We also showed that the mRNA expression level of *α-gustducin* was not changed during 0−9 weeks. This result was identical to that of a previous report showing that chicken taste bud numbers are stable post-hatching (Kudo *et al*., 2008). The present study suggests that one of the functional bitter taste receptors, *cT2R1*, is specifically reduced in the chick palate with growth. Because salty and sour taste sensitivities decrease with aging in chickens (Berkhoudt, 1985), their salty and sour taste receptors may be reduced with growth. Further studies are needed to confirm this and to examine whether sweet and umami taste qualities are affected by growth in chicks. Furthermore, because we used both male and female chicks randomly, we did not confirm the effects of sex on the bitter taste sensitivity in this study. In particular, females are industrially important as egg layers. Further studies are needed to reveal whether sex differences affect taste sensitivities during growth in chicks.

It has been reported that the postnatal administration of the bitter tastant quinine to chicks increases *cT2R1* and *cT2R7* mRNA expression in the palate (Cheled-Shoval *et al*., 2014). This result showed the possibility that the experiences of tastant intake could affect taste receptor expression in chick oral tissues. In the present study, chicks had eaten normal commercial feed from the time of hatching until they were older. Because normal commercial feed does not contain strongly bitter substances, the bitter taste experience of these
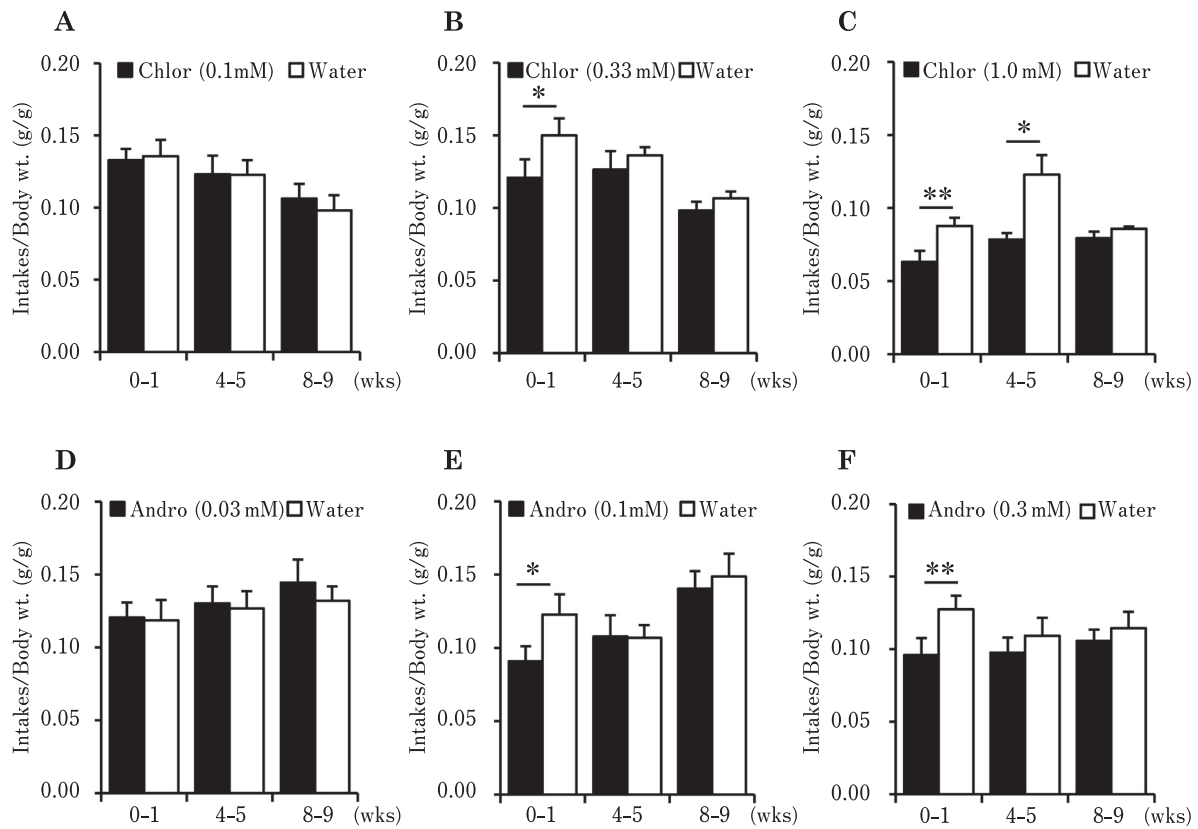
Fig. 1. **Behavioral analyses of the aversive responses to chloramphenicol (Chlor) or andrographolide (Andro) in 0–1-week-old, 4–5-week-old, and 8–9-week-old chicks.** The intakes of 0.33 and 1.0 mM Chlor solutions were significantly lower than the intakes of water in the 0–1-week-old chicks, whereas the 4–5-week-old chicks consumed a significantly lower amount of 1.0 mM Chlor solution than water in a 10-min drinking test (A–C). However, the 8–9-week-old chicks failed to detect the bitterness of the Chlor solutions (A–C). The 0–1-week-old chicks consumed significantly lower amounts of 0.1 and 0.3 mM Andro solutions than water (E, F). On the other hand, the 4–5-week-old and 8–9-week-old chicks did not show any significant aversion to the Andro solutions in comparison with water (D–F). Data are the means$\pm$ SE ($n=8$). $*P<0.05$ and $**P<0.01$ by paired *t*-test.

chicks was not extensive under the present experimental conditions. The reduction of *cT2R1* expression observed in this study may have been induced by the lower level of experience with bitter taste. If taste receptor expression levels in chickens could be changed using various tastants, then their taste sense could be adjusted, which would be very useful in the chicken industry. Interestingly, it has been reported that tumor necrosis factor (TNF) regulates bitter taste responses in mice (Feng *et al.*, 2015). TNF knockout mice showed less sensitivity to bitter compounds than wild-type mice did in behavioral tests. If TNF is decreased with growth in chicks, the decreases in *cT2R1* expression and sensitivity to bitter compounds observed in this study may have been induced by the decrease in TNF. It is important to reveal factors that can modify taste sensitivity in chickens.

In conclusion, we have shown that younger chicks have greater aversion to bitter compounds, and they have significantly higher expression levels of one of the bitter taste receptors than older chicks have. These findings will play a significant role in chicken feed formulation and will help us to widen the scope of taste research in other avian species.
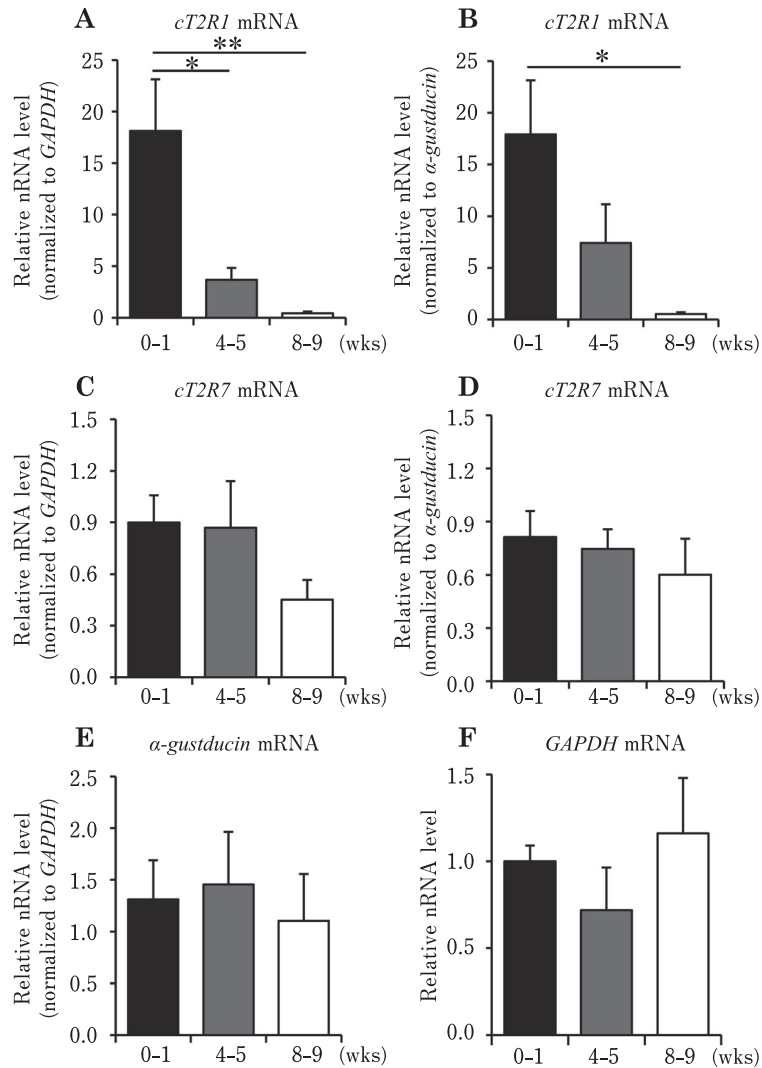
Acknowledgments

Fig. 2. **Relative mRNA levels of bitter taste receptors and α-gustducin in the palates of chicks at three different growth stages, as determined by real-time PCR.** α-Gustducin was used as a positive control for taste cells, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control in this experiment. The 0−1-week-old chicks had a significantly higher amount of *cT2R1* mRNA than that of the 4−5-week- and 8−9-week-old chicks (A, B). However, the relative expression of *cT2R7* mRNA was not statistically different among the chicks of different growth stages, although a declining trend was observed in the 8−9-week-old chicks (C, D). The relative expression levels of α-gustducin mRNA did not differ among the chicks of different growth stages (E). The mRNA levels of *GAPDH* were not significantly different among the chicks of different growth stages (F). Data are the means$\pm$SE ($n=5$, duplicate samples). * $P < 0.05$ and ** $P < 0.01$ by Tukey's test.

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