

# MOLECULAR AND CELLULAR BIOLOGY

## Distribution of $\alpha$ -transducin and $\alpha$ -gustducin immunoreactive cells in the chicken (*Gallus domesticus*) gastrointestinal tract

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**ABSTRACT** The expression and distribution patterns of the taste signaling molecules,  $\alpha$ -gustducin ( $G_{\alpha\text{gust}}$ ) and  $\alpha$ -transducin ( $G_{\alpha\text{tran}}$ ) G-protein subunits, were studied in the gastrointestinal tract of the chicken (*Gallus domesticus*) using the immunohistochemical method.  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{tran}}$  immunoreactive (-IR) cells were observed in the mucosal layer of all examined segments, except the esophagus, crop, and the *saccus cranialis* of the gizzard. The highest numbers of  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{tran}}$ -IR cells were found in the proventriculus glands and along the villi of the pyloric, duodenum, and rectal

mucosa.  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{tran}}$ -IR cells located in the villi of the jejunum, ileum, and cloaca were much less numerous, while only a few  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{tran}}$ -IR cells were detected in the mucosa of the proventriculus and cecum. In the crypts, IR cells were observed in the small and large intestine as well as in the cloaca.  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{tran}}$ -IR cells displayed elongated (“bottle-” or “pear-like”) or rounded shape. The demonstration of  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{tran}}$  expression provides evidence for taste receptor mediated mucosal chemosensitivity in the chicken gastrointestinal tract.

**Key words:** chemosensing,  $\alpha$ -transducin,  $\alpha$ -gustducin, gastrointestinal tract, chicken

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### INTRODUCTION

The gustatory sense is fundamental for food choice and intake. Bitter and sour indicate potentially poisonous or unhealthful food, salty signals the presence of electrolytes, whereas sweet and umami are typically associated with palatability and indicate nutrients (Drewnowski and Gomez-Carneros, 2000). In addition, taste receptors (**TR**) located in the extra-oral tissue such as the gastrointestinal tract are likely to affect a number of physiological responses, including food intake, induced by luminal contents (Sternini 2007; Sternini et al., 2008; Behrens and Meyerhof, 2011; Depoortere 2015).

Nowadays, the high cost of feed in animal production has led to an attempt to include alternative grains and feedstuffs, such as products from human food processing and biofuel production (Godfray et al., 2010; Boland et al., 2013). In this context, a critical question in animal nutrition is how mechanisms governing taste perception interact with food intake behavior.

In vertebrate taste buds, sweet, umami, and bitter tastes are detected by G protein-coupled receptors, known as TR, which interact with specific G protein subunits, including  $\alpha$ -gustducin ( $G_{\alpha\text{gust}}$ ) and  $\alpha$ -transducin ( $G_{\alpha\text{tran}}$ ). These messengers activate several effector systems leading to intracellular  $\text{Ca}^{2+}$  increase and transmitters release (Adler et al., 2000; Matsunami et al., 2000; Behrens and Meyerhof, 2011). Expression of TR and their signalling molecules has been reported in several extra-oral sites including the digestive systems of mammals (Rozenfurt et al., 2006; Sutherland et al., 2007; Janssen et al., 2011; Daly et al., 2012; Widmayer et al., 2012; Mazzoni et al., 2013), chicken (Cheled-Shoval et al., 2014; 2015) and fish (Latorre et al., 2013; Mazzoni et al., 2015), supporting the concept that there is more than a “taste” function for these molecules and that taste receptor-related molecules exert non-gustatory functions outside the mouth (Sternini, 2007; Behrens and Meyerhof, 2011; Finger and Kinnamon, 2011).

The gastrointestinal (**GI**) tract is a sensory organ that responds to a variety of signals originating in the lumen, including nutrient and non-nutrient chemicals, mechanical factors, microorganisms, and toxins. The presence of  $G_{\alpha\text{gust}}$  and/or  $G_{\alpha\text{tran}}$  immunoreactive (-IR)

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cells in the GI epithelial layer, predominantly in enteroendocrine cells (Rozenfurt et al., 2006; Sutherland et al., 2007; Janssen et al., 2011; Daly et al., 2012; Widmayer et al., 2012; Janssen and Depoortere, 2013; Latorre et al., 2013; Mazzoni et al., 2013; Mazzoni et al., 2015), suggests that taste signaling molecules affect GI functions and feeding behavior upon activation through release of substances (e.g., hormones) with an orexigenic and anorexigenic action (Mazzoni et al., 2013). In the present study, we used immunohistochemistry to determine the cellular sites of expression of  $G_{\alpha_{\text{gust}}}$  and/or  $G_{\alpha_{\text{tran}}}$ -IR and assess the density of  $G_{\alpha_{\text{gust}}}$  and/or  $G_{\alpha_{\text{tran}}}$ -IR cells throughout the chicken GI tract (including cloaca). Our study provides an accurate qualitative and quantitative cellular mapping of the taste receptor signaling molecules,  $G_{\alpha_{\text{gust}}}$  and  $G_{\alpha_{\text{tran}}}$ , in the chicken GI tract, extending previous observation of their mRNA tissue distribution. The results of the present study provide a molecular and morphological basis for a role of taste-related molecules in the gut chemosensitive processes of the chicken.

## MATERIALS AND METHODS

A total of 10 one-day-old Ross 308 male chicks were reared on a pen floor using wood-shavings as litter material and fed a commercial diet. At 40 d of age, 4 chickens with a homogeneous body liveweight ( $2.8 \pm 0.1$  kg) were collected and suppressed by cervical dislocation according to the legislation on the protection of animals used for scientific purposes (Directive 2010/63/EU).

The alimentary tract from the esophagus to the cloaca was carefully excised. Specimens of the GI tract, including the oesophagus (*pars cervicalis* and *pars thoracica*), ingluvies or crop, *proventriculus gastris* (in the middle part of the *regio glandularis*), gizzard (in the *saccus cranialis* outside the *ostium ventriculopyloricum* and *pars pylorica*), duodenum (*ansa duodeni*), jejunum (*diverticulum vitellinum* or Meckel's diverticulum), ileum (between ileocecal ligament), cecum (in the *corpus ceci*), rectum (between the ileum and cloaca), and cloaca (urodeum compartment), were collected (the anatomical nomenclature is from Nomina Anatomica Avium) (Baumel et al., 1993). After a brief washing with 0.01 M phosphate buffer saline (PBS), tissues were fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2) for 48 h at 4°C. Fixed tissues were dehydrated in a graded series of ethanol and embedded in paraffin. Transverse paraffin sections were cut at 5  $\mu\text{m}$  thickness on a microtome. To avoid counting the same cells in serial sections, each seventh section was mounted onto poly-L-lysine-coated slides for immunohistochemistry.

### Immunohistochemistry

For single immunofluorescence, sections were deparaffinized in xylene and rehydrated through graded

ethanols. To unmask the antigenic sites, slides were heated in sodium citrate buffer (pH 6.0) in a microwave and subsequently incubated for 30 min in a humid chamber at room temperature in PBS containing 10% normal goat serum, to prevent non-specific bindings. Slides were incubated overnight at 4°C with the following primary antibodies: rabbit anti- $G_{\alpha_{\text{tran}}}$  1:50 (sc-390, Santa Cruz, CA) or rabbit anti- $G_{\alpha_{\text{gust}}}$  1:50 (sc-395, Santa Cruz, CA). After washing, sections were incubated at room temperature for 1 h with FITC-conjugated goat anti-rabbit IgG secondary antibody 1:1500 (Calbiochem, Darmstadt, Germany). Finally, the sections were then washed in PBS and cover-slipped with buffered glycerol, pH 8.6.

### Antibody Specificity

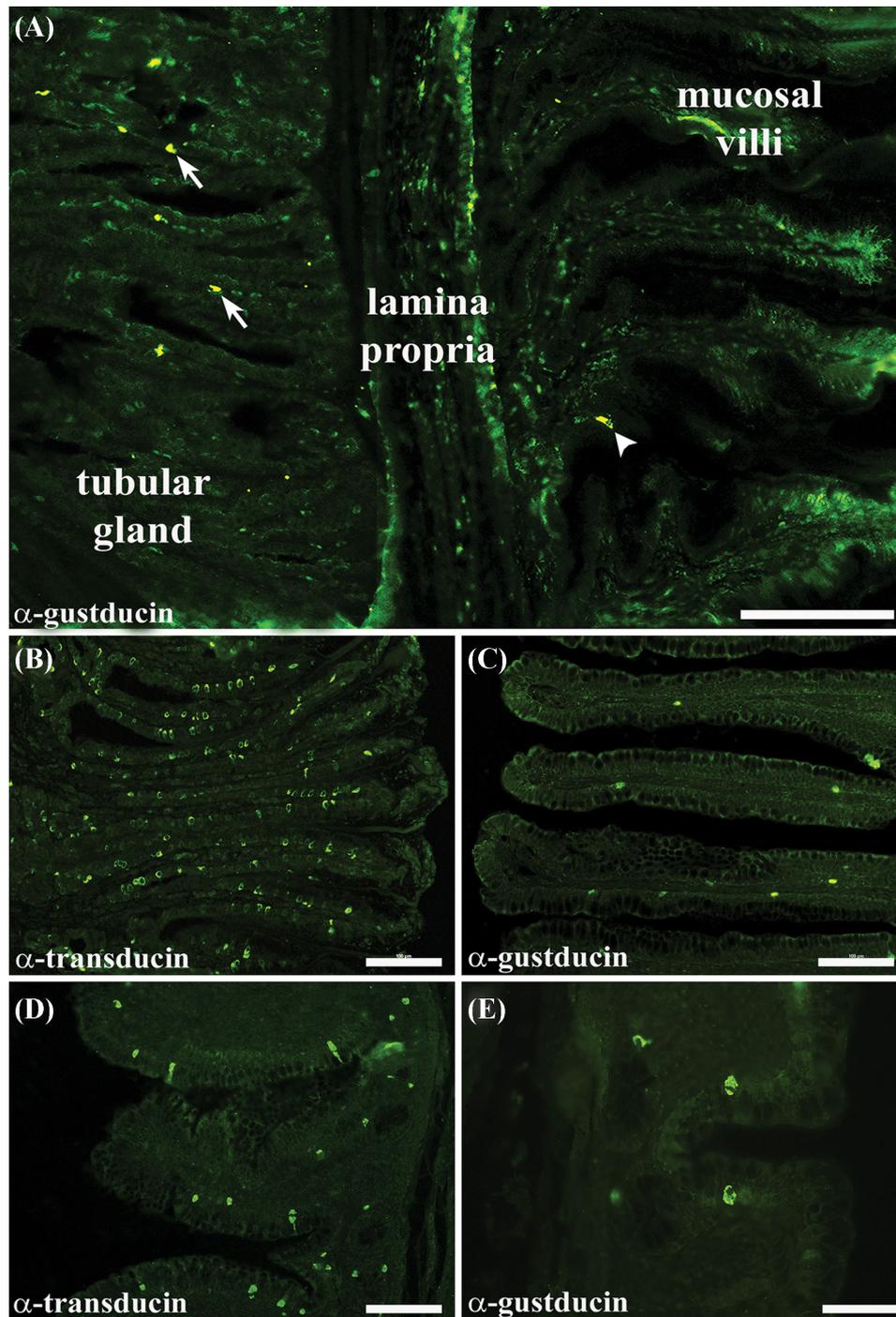
In this study, the specificity of the primary antibodies was evaluated by means of the pre-adsorption test with an excess (20  $\mu\text{g}$  peptide in 1 mL PBS) of the homologous peptides (sc-390P and sc-395P Santa Cruz, CA, respectively). In addition, further specificity tests concerned the secondary antibody and were performed by omitting the primary antibodies. These data are not shown.

### Quantitative Analysis of $G_{\alpha_{\text{gust}}}$ - and $G_{\alpha_{\text{tran}}}$ -IR Cells

Cell counting was performed with a 40 X objective lens using a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany) with appropriate filter cubes to discriminate different wave fluorescence. Images were collected with a Polaroid DMC digital photocopier (Polaroid, Cambridge, MA) and minimal adjustment to brightness and contrast was performed with Corel Photo Paint and Corel Draw (Corel, Dublin, Ireland). Cell counting was performed in a blind fashion by 2 investigators (M. M. and C. V.). For each chicken,  $G_{\alpha_{\text{gust}}}$  and  $G_{\alpha_{\text{tran}}}$ -IR cells were counted in 30 random villi (or folds) and, when they were present, in 30 intestinal glands (crypts) in the GI tract. In the proventriculus, as well as in endoluminal epithelium, positive cells also were observed in the simple tubular glands of the *lamina propria*; for this reason the glandular IR cells were counted in 18 random microscope fields (0.28 mm<sup>2</sup> each field), for a total area of 5 mm<sup>2</sup>. Only villi/glands/crypts located perpendicularly to the mucosal surface were counted. The values were pooled for each GI collected tract (proventriculus, gizzard *pars pylorica*, duodenum etc.) and expressed as mean  $\pm$  standard deviation (SD).

## RESULTS

$G_{\alpha_{\text{gust}}}$  and  $G_{\alpha_{\text{trans}}}$ -IR cells were detected throughout the whole chicken GI tract (Figures 1A–1E), except for the esophagus (proximally and caudally to the



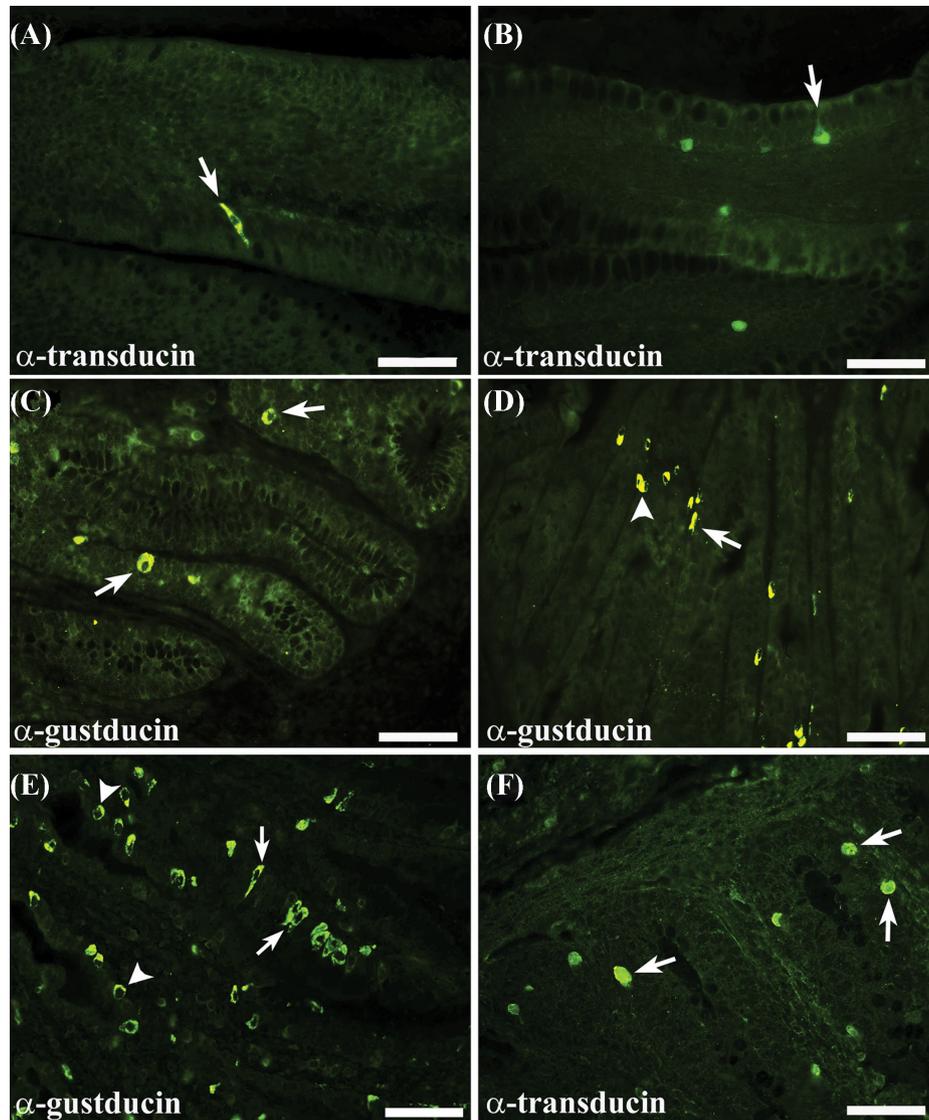
**Figure 1.** Localization of  $G_{\alpha\text{gust}}$ - and  $G_{\alpha\text{trans}}$ -IR cells in the chicken GI tract. (A) shows  $G_{\alpha\text{gust}}$ -IR cells located both in the endoluminal epithelium (arrowhead) and in the tubular glands (arrows) of the proventriculus. (B–E) images show  $G_{\alpha\text{gust}}$ - or  $G_{\alpha\text{trans}}$ -IR cells in the pyloric (B), ileum (C), rectal (D), and cloaca (E) mucosa. A–E scale bars = 50  $\mu\text{m}$ .

crop), crop, and the *saccus cranialis* of the gizzard. The positive cells displayed a “bottle-like” shape with a central nucleus and 2 cytoplasmic prolongations oriented in opposite directions: the first directed towards the mucosal surface, the second to the basal lamina (Figure 2A). In other cases, the IR cells showed a “pear-like” shape with an unlabelled nucleus and the base near the basal lamina. These cells possess a cytoplasmic prolongation that passes through the epithelial lining and appears to reach the lu-

menal surface (Figure 2B). Finally, there are some rounded shape IR cells confined at the basal lamina, which do not appear to reach the lumen (Figure 2C).

### Proventriculus

In the glandular stomach,  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$  labeled cells were observed in both the endoluminal epithelium and close to tubular glands (Figure 1A): These positive



**Figure 2.** Photomicrographs of  $G_{\alpha\text{gust}}$ - or  $G_{\alpha\text{trans}}$ -IR cells in the duodenum (A), jejunum (B), rectum (C), proventriculus (D), pyloric (E), and cecum (F) mucosa of chicken. In general,  $G_{\alpha\text{trans}}$ -IR and  $G_{\alpha\text{gust}}$ -IR cells observed along the villus surface show an elongated (A, arrow) or pear-like (B, arrow) shape. In other cases, the labeled cells observed at the basal lamina of the glands exhibited a rounded (C, arrows) shape. In the pyloric mucosa and in proventriculum glands, elongated (arrows) and rounded (arrowheads) positive cells (D and E, respectively) were observed simultaneously. In the cecum, labeled cells (rounded shape) were mainly observed in the intestinal glands (F, arrows). A–F scale bars = 50  $\mu\text{m}$ .

cells were bottle-like, alternated with rounded shape (Figure 2D). In the mucosal surface, IR cells were observed in both villi and crypts, while in the simple tubular glands they appeared along the adenomer (more numerous at the base), without any apparent order.

These cells were significantly more abundant in the tubular glands compared to the superficial epithelium, where only very few  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$ -IR cells were counted and their mean numbers were very low (Table 1). In the tubular glands the number of  $G_{\alpha\text{gust}}$ -IR ( $100.8 \pm 7.9$ ) was higher than that of  $G_{\alpha\text{trans}}$ -IR cells ( $84.5 \pm 8.4$ ).

### Gizzard Pyloric Mucosa

$G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$ -IR cells were numerous in the pyloric mucosa (Table 1). These cells were distributed

along the mucosal villi. Notably, elongated or rounded shape cells co-existed in the same villous. Also, while bottle-like or pear-like IR cells were more superficial, the rounded IR cells prevailed in the inner part of the villus epithelial lining (Figure 2E).

The intensity of  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$  positivity showed a trend to decrease and then disappear at the transition between the pyloric and gizzard mucosa corresponding to the cuticle (data not shown).

### Small Intestine

In the small intestine,  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$ -IR cell intensity was higher in the villi compared to the crypts. Positive cells displayed a bottle-like or pear-like shape and occupied the entire thickness of the mucosa

**Table 1.** Mean number of  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$ -IR cells in the chicken GI tract.

	Villus		Crypts	
	$G_{\alpha\text{gust}}$	$G_{\alpha\text{trans}}$	$G_{\alpha\text{gust}}$	$G_{\alpha\text{trans}}$
<b>Proventriculus</b>	2 ± 1	6.7 ± 4.5	–	–
<b>Pyloric Muc.</b>	332 ± 13.9	300 ± 11.4	–	–
<b>Duodenum</b>	184.3 ± 10	177.7 ± 9.3	42.7 ± 4.5	33.3 ± 2.5
<b>Jejunum</b>	41.3 ± 6	37 ± 6.5	22 ± 3	21.3 ± 1.3
<b>Ileum</b>	88.3 ± 7.5	82 ± 8.3	26.3 ± 1.5	27.3 ± 3.6
<b>Cecum</b>	1.7 ± 0.6	1.3 ± 0.6	44.7 ± 1.5	47.7 ± 2.1
<b>Rectum</b>	117.8 ± 10	116.7 ± 3.8	36.3 ± 4.2	32.3 ± 3.3
<b>Cloaca</b>	27.5 ± 3.5	23 ± 8.5	33 ± 4.2	32.3 ± 3.3

Values represent the mean number of IR cells evaluated in 30 villi and in 30 intestinal glands. Results were expressed as mean ± standard deviation (SD).

(Figures 2A, 2B). In the duodenum the highest number of positive cells was visualized in the villi compared to the jejunum and ileum. Indeed, in the jejunum the number of positive cells decreased both in the villi and crypts. In contrast, in the ileum  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$  labeled cells increased along the crypt-villus axis without reaching the values observed in the duodenum (Table 1).

### Large Intestine and Cloaca

Positive cells were more abundant in the rectum rather than the cecum and cloaca as shown in Table 1. Generally, positive cells were localized in the intestinal glands and along the surface of the villi. The number of labeled cells in the villi was higher than that of the crypts only in the rectum, while most of the labeled cells, with rounded profile, were detected in the intestinal glands of the cecum (Figure 2F) and cloaca.

## DISCUSSION

The expression of TR and taste-signaling components in the GI tract was first reported more than 20 years ago. The evidence so far acquired suggests that TR and related messengers are involved in metabolic regulation and food intake in the GI tract (Buchan, 1999; Furness et al., 1999; Sternini 2007; Sternini et al., 2008; Behrens and Meyerhof, 2011).

The results of the present study demonstrate the presence of  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$ -IR cells throughout the chicken GI tract with the exception of the esophagus, crop, and the *saccus cranialis* of the gizzard. These findings are in line with a previous report of taste receptor and downstream effectors (including  $G_{\alpha\text{gust}}$ ) gene expression in the chicken GI tract, including the proventriculus, gizzard, duodenum, jejunum, ileum, cecum, and colon, using RT-PCR analysis (Cheled-Shoval et al., 2015). Our results extend these data by providing information on the cellular sites of expression of taste receptor signaling molecules. However, in our study we observed the highest number of  $G_{\alpha\text{gust}}$ -IR cells in the chicken *pars pylorica* followed by the duodenum and rectum, whereas Cheled-Shoval et al., (2015) reported

high levels of G-agustducin mRNA expression in the gizzard, duodenum, and colon rectum, with the highest expression in the colon rectum. This difference in findings may be explained by the different methods employed (RT-PCR vs. immunohistochemistry) for  $G_{\alpha\text{gust}}$  detection and the different points of sample collections. Indeed, we collected the gizzard in 2 points (*saccus cranialis* outside the *ostium ventriculopyloricum* and *pars pylorica*), while Cheled-Shoval et al., (2015) did not specify the sampling site. By contrast, Kudo et al., (2010) could not detect the expression of the  $G_{\alpha\text{gust}}$ -like sequence in the chicken small intestine.

Overall our results expand previous reports of TR and downstream signaling messengers (including  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{trans}}$ ) along the GI tract in different species (Rozengurt et al., 2006; Daly et al., 2012; Widmayer et al., 2012; Latorre et al., 2013; Mazzoni et al., 2013; Cheled-Shoval et al., 2014; 2015; Mazzoni et al., 2015). Moreover, the present results are consistent with those obtained by our group (Mazzoni et al., 2013) in the pig GI tract where we found many  $G_{\alpha\text{trans}}$ -IR cells in the pyloric and duodenal mucosa. Furthermore, in the sea bass (Latorre et al., 2013) and sole (Mazzoni et al., 2015), the highest number of  $G_{\alpha\text{trans}}$ -IR and/or  $G_{\alpha\text{gust}}$ -IR cells was in the stomach. These results suggest a pivotal role of the upper part of the GI tract in the chemosensory processes. In this regard, Widmayer et al., (2012) suggested that the stomach has an ideal and strategic position for monitoring food tastes in order to detect potentially harmful substances and activate digestive processes. Likewise, Akiba and Kaunitz (2011) indicated that the duodenum, located between the pylorus and the pancreaticobiliary ducts, also could exert a crucial role in the luminal chemosensing. On the other hand, Cheled-Shoval et al., (2015) hypothesized that the presence of high levels of bitter TR and their signaling G-protein  $G_{\alpha\text{gust}}$  in the chicken colon could imply that the bitter taste pathway is involved in bacterial homeostasis and pathogen detection. This is in line with previous data from our group showing up-regulation of  $G_{\alpha\text{gust}}$  and TR subtype expression in the mouse colon in response to a long-term high-fat diet associated with changes of gut microbiota in the colon (Vegezzi et al., 2014). In their review, Kaji et al. (2014) proposed that sensory receptors expressed in the colonic mucosa, including TR, play a key role in luminal chemosensing by detecting bacteria and their metabolites and contributing to the maintenance of body homeostasis.

The morphological aspect of  $G_{\alpha\text{gust}}$ - and  $G_{\alpha\text{trans}}$ -IR cells, which displayed elongated (bottle-like or pear-like) or rounded shape morphology (Sutherland et al., 2007, Mazzoni et al., 2013, Mazzoni et al., 2015) suggests that they are of endocrine nature (i.e., “open-” and “closed-type” enteroendocrine cells). Neglia et al., (2005) observed a high number of small, round-shaped (considered closed-type cells) ghrelin-IR cells compared to few cells with an elongated shape and apical cytoplasmic processes in contact with the lumen (open-type cells) in the chicken GI tract. Also, D’Este and Renda

(1995) showed histamine endocrine bipolar elongated shaped cells with their main axis parallel to the septa glandular and with the appearance of close-type cells in the proventricular glands. In the chicken GI tract (gizzard, antrum, small and large intestine, and cloaca), Salvi et al., (1995; 1996) found open-type chromogranin A- and B-IR cells, whereas roundish, closed-type cells were located in the inner part of the mucosa. Finally, in the chicken ileum (Watanabe et al., 2014) and small intestine (Monir et al., 2014) glucagon-like peptide- 1 and -2-IR have been reported in “flask-like” (reminiscent of open-type) enteroendocrine cells. All together, these studies provide evidence for the presence of different types of enteroendocrine cells in the chicken GI tract.

In summary, our results showing the localization of  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{tran}}$ -IR in epithelial cells with the morphology of open- and close-type enteroendocrine cells in the chicken GI mucosa extend previous reports of TR signaling molecule mRNA, including  $G_{\alpha\text{gust}}$  mRNA, expression in the chicken GI tract (Cheled-Shoval et al., 2015). The cellular localization of TR signaling molecules supports a role of taste signaling machinery in poultry gastrointestinal chemosensitivity. Further studies are required to characterize the neuroendocrine profile of  $G_{\alpha\text{gust}}$  and  $G_{\alpha\text{tran}}$ -IR cells.

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