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Title: Molecular cloning of growth hormone secretagogue-receptor and effect of quail ghrelin on gastrointestinal motility in Japanese quail

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Keywords: Chicken, Japanese quail, Ghrelin, Growth hormone secretagogue-receptor, Gastrointestinal tract, Motilin

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Abstract: We identified a growth hormone secretagogue-receptor (GHS-R) for ghrelin (GRLN) in the Japanese quail, and examined relationship between its receptor distribution and the effects of ghrelin on the gastrointestinal tract of the quail. GHS-R expression and GRLN-induced response were also investigated in the chicken and compared with quail. Several types of GHS-R, namely GHS-R1a-L, GHS-R1a-S, GHS-R1aV, GHS-R1b, GHS-R1bV and GHS-R1tv-like receptor, were identified in quail cerebellum cDNA. Amino acid sequence of quail GHS-R1a-L was 98% identical to that of chicken GHS-R1a. GHS-R1a mRNA was expressed heterogeneously in the quail gastrointestinal tract with a high expression level in the colon, moderate levels in the esophagus and crop, and low levels in the proventriculus, gizzard and small intestine. The region-specific expression pattern was almost the same as that in the chicken. Chicken and quail GRLN caused contraction in the crop, proventriculus and colon of both the quail and chicken, whereas the small intestine was less sensitive. However, the contractile efficacy was more potent in the chicken than in the quail. Chicken motilin (MTL), another gut peptide, structurally resemble to GRLN, caused

marked contraction in the small intestine of both the quail and chicken, and the region-specific effect of MTL was opposite to that of GRLN. In conclusion, GRLN mainly induces the contractile responses of the upper and lower gastrointestinal tract and MTL stimulates motility of the middle intestine in both the quail and chicken. Regions in which GRLN acts were consistent with the distribution of GHS-R1a mRNA, but the contractile efficacy was different in the quail and chicken. These results suggest a species-specific contribution of GRLN in the regulation of avian gastrointestinal contractility.

July 3th, 2009

Prof WE Schmidt
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Re: REGPEP-D-09-00073

Dear Dr. Schmidt

We are re-submitting our manuscript to you for consideration of its possible publication in *Regulatory Peptides*, TITLE: **Molecular cloning of growth hormone secretagogue-receptor and effect of quail ghrelin on gastrointestinal motility in Japanese quail,** by Kitazawa et al.

Thank you for the reviewers' useful comments on our manuscript. Considering the comments, we have revised the manuscript. I am a little bit afraid that the referees satisfy our revision but I did my best in this manuscript. Please check the responses to the reviewers' comments and the revised version of the manuscript.

Thank you for considering this paper for possible publication in *Regulatory Peptides.*

Sincerely,

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Responses to Reviewer's Comments

We sincere express our gratitude for the reviewer's comments to improve our manuscript. Point-by-point answers for the comments and queries are described below

Comments of the Reviewer1

Q: The authors made a mistake in the graph legends for chicken ghrelin (¦) and quail ghrelin (?) in Figure 4. This should be corrected. The results in this figure showed a remarkable difference in changes in intracellular Ca2+ concentrations between chicken and quail ghrelin treatment. An explanation or discussion of this difference from the standpoint of the structure-activity relationship for chicken and quail ghrelin is important for readers and should be included in the discussion section.

A: We thank the reviewer for picking up this mistake. We have changed the legend for Figure 4. Before doing the experiments, we expected that quail GRLN could be more effective to quail GHS-R than chicken GRLN. However, interestingly, the result is opposite: quail GRLN was less effective than chicken GRLN. It is responsible for difference in amino acid sequence of peptides: amino acid sequence between quail and chicken GRLN differs at three positions (8, 17, 22), the differences in chicken GRLN might affect positively the binding to the quail GHS-R and subsequent increase in Ca^{2+} concentration, despite of the heterologous system. In addition to this, physiological significance of the different responsiveness has been unclear yet, we postulate that quail may be a low sensitive to GRLN compared with chicken because of the present results. Different responses of GRLN between chicken and quail observed in previous food intake study and the current GI contraction study suggest species-specific physiological roles of GRLN in either quail or chicken (P26, L445-454, P42 Figure 4 legend).

Minor comments

1) Locations of the companies should be stated in the manuscript. P8, L105, P8, L124 and P9, 139.

A: Name and location of animal service companies have been added in the revised version (P7 L107-108). Company names have been indicated at the first time when it appeared in the text. In the case of P8 L124, Invitrogen has already described (P7 L121).

2) The accession numbers of genes in the legend of Fig. 2 should also be stated in the Materials and Methods section of the manuscript.

A: We agreed with the reviewer's suggestion and the accession numbers of genes have been stated in the Methods section of the manuscript (see Materials in the revised MS).

3) P24, L413-414: A report concerning the natural knockout of rodent motilin should be quoted. (e.g., Peeters, Neurogastroenterol Motil 2004;16:687, Aerssens J, Neurogastroenterol Motil 2004;16:841)

A: As the reviewer mentioned, references concerning the natural knockout of motilin and motilin receptor genes are needed. We have inserted two references that the reviewer had recommended (P25 L436, reference number 25, 26 in the revised MS).

4) P37: Primer concentrations in Table 1 are complicated. Moving these descriptions to Materials and Methods is recommended.

A: We agreed the reviewer suggestion, and the primer concentrations were described in the Methods section and the Table 1 has been changed (see Materials and Methods, Table 1).

5) Notations should be unified. P28, L488: MTL receptor (MTL-R) and P7, L96: MTL receptor (GPR38).

A: "MTL receptor" has been used throughout the revised version (P30L518-519).

6) The authors used the term "affinities to the receptors" in the manuscript (P20, L336). However, in this study, the authors investigated "the effect on changes of intracellular Ca2+", not "receptor affinity". This should be rewritten.

A: As the reviewer pointed out, we had not done the binding study. Therefore, "affinity" is not correct expression. We used "effective or effectiveness" in the revised version (P21 L357-359).

Comments of the Reviewer-3:

1) In the abstract line 18: ... and examined firstly the relationship between... and secondly the effects on the...

A: As the reviewer pointed out, this sentence was a little bit complicated. We have divided this section into two sentences to make it clear (P2 L17-20).

2) line 19: ...the effects in the chicken...

A: This section revised according to the comment (P2 L17-20).

3) line 27: ...and colon of both the quail and chicken, whereas the small intestine...

A: This section revised according to the comment (P2 L28-29).

4) lines 48-50: "GRLN is an important ... gastric acid secretion [2]" needs rephrase

A: This section has been a little complicated. We have rephrased the sentence (P4 L49-51)

5) line 77: has been observed in another bird, the Japanese quail [13]. Interestingly it is observed that this effect is opposite in mammals and some teleost...

A: As the reviewer pointed out, this section was complicated. We revised the sentence in the revised version (P5-6 L77-80)

6) line 79: delete Thus... , start with "Birds are..."

A: “Thus” was deleted and start “Birds are…” (P6 L80)

7) line 80: delete However..., start with "Furthermore, it is interesting..."

A: We agreed the reviewer comment. Instead of “However”, “Furthermore” has been used in the revised version (P6 L81)

8) line 81-82: quail: GRLN in low dose stimulates food intake, whereas in high dose inhibits food intake [19].

A: We agreed the reviewer comment and this was revised (P6 L82-81)

9) line 90: in the Japanese quail

A: We have revised “in the” before Japanese quail (P6 L91).

10) line 92:In addition, it was investigated the relationship between tissue distribution... mechanical response to GRLN in different parts of the....

A: We have changed this section following the comment (P6 L93-94).

11) line 98: tract of the quail and it was compared to the GRLN's effects.

A: This section has been revised as follow “the gastrointestinal tract of both the chicken and the quail and it was compared to the GRLN-induced responses. (P7 L98-100)

12) lines 345-6: rephrase the "in whole intact tissue were also measured" (???)

A: As the referee pointed out, the meaning of this section was unclear. We have rephrased as follow “mRNA expression levels in whole gastrointestinal tract as well as in smooth muscle

layers without mucosa were also measured" (P22 L367-368).

13) line 372 "than that of chicken GRLN -induced in the chicken" (??) what do you mean?

A: As indicated in Fig. 6, when the responsiveness was compared in the homologous system such as quail GRLN to quail GI and chicken GRLN to chicken GI, we could observe much stronger effect in the chicken system than in the quail system, in terms of GI contractile response. Fig. 7 indicated the value of relative contraction induced by GRLN in the respective birds. This section revised more precisely in the revised version (P23, L393-395).

14) line 399: also caused contraction ...

A: "contraction" has been used in the revised manuscript (P25 L421).

15) DISCUSSION line 405-408: In the present study, we identified GHS-R in the Japanese quail and examined the extent of GHS-R1a mRNA distribution in the Japanese quail gastrointestinal tract and the effect of GRLN on the motility of the gastrointestinal tract. GHS-R 1a expression and GRLN-induced responses were also investigated in the chicken and compared with quail.

A: We agreed with the reviewer's suggestion. The part was revised (P25 L427-431).

16) line 418: instead of "and the long type and short type..." prefered "while or whereas the long type and..."

A: Instead of "and", "while" has been used in the revised manuscript (P26 L440)

17) line 430: there has not been any report on..., not even in the chicen; but..."

??

A: We agreed the reviewer comment, and this section has been revised (P27 L459-460)

18) line 448: Furthermore, as previously reported, we identified a cDNA... as GHS-R1tv [13].

A: We agreed the reviewer comment, and this section has been revised (P28 L477)

19) line 469: GRLN-induced contraction was ...

A: We agreed the reviewer comment, and this section has been revised (P29 L498)

20) Figure 3 legend: too big. is it possible to be shrieked?

A: We have included almost all explanation in the revised manuscript (P20-21, Figure 3 legend). Fig. 3 has been a little bit changed for better understanding of the data.

Molecular cloning of growth hormone secretagogue-receptor and effect of quail ghrelin on gastrointestinal motility in Japanese quail

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Abstract

We identified a growth hormone secretagogue-receptor (GHS-R) for ghrelin (GRLN) in the Japanese quail, and examined relationship between its receptor distribution and the effects of ghrelin on the gastrointestinal tract of the quail. GHS-R expression and GRLN-induced response were also investigated in the chicken and compared with quail. Several types of GHS-R, namely GHS-R1a-L, GHS-R1a-S, GHS-R1aV, GHS-R1b, GHS-R1bV and GHS-R1tv-like receptor, were identified in quail cerebellum cDNA. Amino acid sequence of quail GHS-R1a-L was 98% identical to that of chicken GHS-R1a. GHS-R1a mRNA was expressed heterogeneously in the quail gastrointestinal tract with a high expression level in the colon, moderate levels in the esophagus and crop, and low levels in the proventriculus, gizzard and small intestine. The region-specific expression pattern was almost the same as that in the chicken. Chicken and quail GRLN caused contraction in the crop, proventriculus and colon of both the quail and chicken, whereas the small intestine was less sensitive. However, the contractile efficacy was more potent in the chicken than in the quail. Chicken motilin (MTL), another gut peptide, structurally resemble to GRLN, caused marked contraction in the small intestine of both the quail and chicken, and the region-specific effect of

MTL was opposite to that of GRLN. In conclusion, GRLN mainly induces the contractile responses of the upper and lower gastrointestinal tract and MTL stimulates motility of the middle intestine in both the quail and chicken. Regions in which GRLN acts were consistent with the distribution of GHS-R1a mRNA, but the contractile efficacy was different in the quail and chicken. These results suggest a species-specific contribution of GRLN in the regulation of avian gastrointestinal contractility.

Key words: Chicken, Japanese quail, Ghrelin, Growth hormone secretagogue-receptor, Gastrointestinal tract, Motilin

1. Introduction

Ghrelin (GRLN), a 28-amino-acid peptide in which the third serine residue (Ser^3) has an *n*-octanoyl modification, is an endogenous ligand for the growth hormone secretagogue-receptor (GHS-R) that was identified in rats and humans [1]. GRLN was first identified as a growth hormone (GH)-releasing peptide that is mainly produced in the stomach, but accumulating evidences indicate that GRLN is an important hormone to regulate glucose metabolism, feeding, cardiovascular function and gastrointestinal function (motility and gastric acid secretion) [2]. The multifunctional roles of GRLN are supported by wide expression of mRNA and protein for GHS-R from the central nervous system to several peripheral tissues [2-4]. In mammals, two GHS-R isoforms, a functional receptor GHS-R1a, and an alternative splice variant, GHS-R1b (not functional), have been identified [5].

GRLN has been identified in many species of non-mammalian vertebrates [6, 7]. In the chicken, GRLN is composed of 26 amino acids, and Ser^3 has been modified by *n*-octanoic or *n*-decanoic acid [8]. Chicken GRLN shares about 50% total sequence identity to human GRLN and 100% identity to the N-terminal region (Gly^1-Pro^7). Chicken GRLN mRNA is predominantly expressed in the proventriculus [6, 8, 9]. Quail

GRLN structure, composed of 26 amino acid, shares 88% identity to chicken GRLN. Quail GRLN mRNA and protein expression have been demonstrated in the proventriculus and oviducts [10].

Distribution and characterization of chicken GHS-R have been already reported [11, 12]. Two types of GHS-R were found in the chicken: GHS-R1a is considered as a functional receptor, and GHS-R1aV (GHS-R1c) is the splice variant where 16-amino acids (48 bp) in transmembrane-6 are lacking. Sirotkin et al. [13] reported another splice variant, GHS-R1tv, that is specifically expressed in the gonad. GHS-R1a mRNA expression has been detected in many central tissues (pituitary, hypothalamus, telencephalon, cerebellum and brainstem) and peripheral tissues (ovary, kidney, proventriculus, duodenum and colon) [11, 12]. The wide expression of GHS-R1a mRNA in chicken organs suggests that GRLN exerts multiple physiological functions through binding to the receptors, as has been observed in mammals.

In the chicken, GRLN stimulates the release of GH and corticosterone as an endocrine function [8]. GRLN also regulates appetite in the chicken, and intracerebroventricular (ICV) and intravenous (IV) injections of GRLN have been shown to suppress food intake [14-16]. An inhibitory effect of ICV injection of GRLN has also been observed in another bird, the Japanese quail [13]. Interestingly, the effect

79 observed in both birds is opposite to mammals and a teleost goldfish, in which GRLN
80 stimulates food intake [17, 18]. Birds are the only animals in which an inhibitory effect
81 of GRLN on food intake has been reported. Furthermore, it is interesting to note that
82 peripheral injection of GRLN shows two different actions in the Japanese quail: GRLN
83 in low dose stimulates food intake, whereas in high dose inhibits food intake [19]. Since
84 gastrointestinal motility is relevant to feeding [20] and plasma GRLN level has been
85 shown to be affected by feeding [2], it would be interesting to compare the effect of
86 GRLN on contractility of the gastrointestinal tract in the chicken and quail. We have
87 already shown that chicken GRLN caused region-specific contraction in the isolated
88 chicken gastrointestinal tract and was more effective in the upper and lower
89 gastrointestinal tract than in the middle intestine [21].

90 The main purpose of this study was to compare the effects of GRLN on
91 contractility of the gastrointestinal tract in the chicken and in the Japanese quail. We
92 first characterized GHS-R in the Japanese quail to examine its distribution in the
93 gastrointestinal tract. In addition, it was investigated that the relationships between
94 tissue distribution of GHS-R mRNA and mechanical responses to GRLN in different
95 parts of the quail and chicken gastrointestinal tract. Motilin (MTL) is another gut
96 peptide that is composed of 22 amino acids and is structurally related to GRLN [22, 23].

GHS-R is homologous to the MTL receptor (GPR38) in several points [22-24]. Thus, chicken MTL-induced contraction was also examined in several regions of the gastrointestinal tract of both the chicken and quail, and it was compared to the GRLN-induced response.

2. Materials and methods

All experiments were performed in accordance with Institutional Guidelines for Animal Care at Rakuno Gakuen University.

2.1. Animals and tissue preparations

Male white Leghorn chickens (3-6 weeks, Hokuren, Iwamizawa, Japan) and male Japanese quails (5-9 weeks, Sankyo Lab Service, Sapporo, Japan) were used. Both the chickens and quails were anaesthetized with diethyl ether, stunned, and bled to death. The whole brain, esophagus, crop, proventriculus, gizzard, duodenum, jejunum, ileum and colon were removed after a midline incision, and their luminal contents were flushed out using ice-cold Krebs solution. The esophagus, crop, proventriculus and colon were cut open longitudinally, and smooth muscle strips in the longitudinal direction (1 mm in width and 10 mm in length) were prepared. In the case of the small

intestine (duodenum, jejunum and ileum), longitudinal muscle layers were peeled out mechanically using a cotton-wool swab and fine tweezers. Isolated smooth muscle strips were used for both contraction and molecular studies. Each whole gastrointestinal tissue isolated was also used for molecular study.

2.2. cDNA cloning of quail GHS-R

Total RNA was extracted by TRIzol reagent (Invitrogen, Grand Island, NY) from the cerebrum and cerebellum of the Japanese quail and stored in RNAlater (Ambion, Applied Biosystems, Foster City, CA). Full-length cDNA encoding quail GHS-R was determined to amplify an approximately 690-bp fragment using degenerated primers that were designed on the basis of a portion that is highly conserved across GHS-Rs. Then 3'- or 5'-rapid amplification of cDNA end (RACE) PCR was performed, based on the defined nucleotide sequence using the GeneRacer Kit (Invitrogen).

Cerebellum total RNA (2 μg) was reverse-transcribed with GeneRacer 3'-oligo using a QuantiTect RT Kit (QIAGEN GmbH, Hilden, Germany) (final volume of 40 μl). PCR was performed with 2 μl of a template, a primer set (GHS-R-dSES1 [50 pmol/μl] and GHS-R-dANT1 [50 pmol/μl], Table 1) and *ExTaq* DNA polymerase (TaKaRa,

Shiga, Japan). The reaction conditions were 94˚C for 2 min and subsequent 35 cycles of 94˚C for 0.5 min, 53˚C for 0.5 min and 72˚C for 1 min, and final extension was 72˚C for 3 min. The amplified product was purified by a Wizard PCR preps DNA Purification System (Promega, Madison, WI) and subjected to the second-round nested PCR. Nested PCR was performed under the same conditions as those for primary PCR using another primer set (GHS-R-dSES2 and GHS-R-dANT1, 50 pmol/μl each, Table 1), 2 μl PCR-prepsed template and *ExTaq* DNA polymerase. The obtained product was subcloned into the pCRII-TOPO vector (Invitrogen), and the nucleotide sequence of the insert was determined by automated sequencing (Model 3130, Applied Biosystems) according to protocol of the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems).

For 3'-RACE PCR, primary PCR was performed with the gene-specific primer QL-GHSR-S1 (10 pmol/μl) or -S2 (10 pmol/μl) (Table 1) and 3'-primer using *HotSTAR Taq* Plus mix (QIAGEN GmbH). The reaction conditions were 95˚C for 5 min and subsequent 35 cycles of 95˚C for 0.5 min, 60˚C for 0.5 min and 72˚C for 1 min, and final extension was 72˚C for 3 min. After PCR preps of the amplified product, nested PCR was performed with the gene-specific primer QL-GHSR-S3 (10 pmol/μl) or -S4 (10 pmol/μl) (Table 1) and 3'-nested primer under the same conditions as those for

primary PCR. Three bands appeared, and each band was excised from the gel, subcloned, and sequenced.

For 5'-RACE PCR, first-strand cDNAs were synthesized from 5 μg cerebellum total RNA with an anti-sense primer (QL-GHSR-AS1, 10 pmol/μl) or oligo dT_{12-18} primer (Invitrogen) using Superscript III reverse-transcriptase (Invitrogen). Primary PCR was conducted using the gene-specific primer QL-GHSR-AS1 (10 pmol/μl) or -AS4 (10 pmol/μl) (Table 1), 5'-primer and *HotSTAR Taq* Plus mix with amplification conditions at 95˚C for 5 min and subsequent 35 cycles of 95˚C for 0.5 min, 58˚C for 0.5 min and 72˚C for 1.5 min, and final extension was 72˚C for 3 min. After PCR preps of the product, nested PCR was performed using QL-GHSR-AS2 (10 pmol/μl) or -AS3 (10 pmol/μl) (Table 1), 5'-nested primer and *HotSTAR Taq* Plus mix under the same conditions as those for primary PCR. A specific product was obtained from templates synthesized from both gene-specific anti-sense primers (QL-GHSR-AS2 or -AS3) (10 pmol/μl each) or oligo-dT_{12-18} primer. A large amount of the product originating from oligo-dT_{12-18} primer was subcloned and sequenced.

2.3. Partial cloning of quail GHS-R gene

168 For quantitative real-time PCR (qPCR) of quail GHS-R1a (**acc#AB469019)**, we
169 used a primer set (QL-GHSR-S2 and QL-GHSR-AS2, 10 pmol/µl each, Table 1) to
170 obtain a 196-bp product. However, a band other than the 196-bp product appeared even
171 when total RNA was treated with DNase before reverse transcription. Thus, the
172 amplified products were cloned and the insert was sequenced. The product contained
173 partial sequences of quail GHS-R1a at the 5'- and 3'-sides, but unknown nucleotide
174 sequences have been inserted in the middle portion. We therefore conducted genomic
175 PCR using the same primer set with *PrimeSTAR* DNA polymerase (TaKaRa). Template
176 genomic DNA was obtained from the proventriculus using a Genomic Preps Cell and
177 Tissue DNA Isolation Kit (GE Healthcare, Buckinghamshire, England). The
178 amplification conditions were 98˚C for 1 min and subsequent 35 cycles of 98˚C for 20
179 sec, 55˚C for 30 sec and 72˚C for 20 sec. An approximately 2.5- kbp product was
180 subcloned into the pCRII-TOPO vector after overhang reaction with *ExTaq* DNA
181 polymerase (TaKaRa). For the sequencing, two sequencing primers (quailGHSR-int-s1:
182 5'-TCA GCC TTT GCT GAA CAG TGA CCA-3', and quailGHSR-int-AS1: 5'-ACA
183 AAG GCT ACA TGC AAT TTA TGG-3') were designed.
184

185 **2.4. Functional analysis of quail GHS-R**

We found two candidates for an open reading frame in quail GHS-R1a cDNA. Two primer sets to amplify each GHS-R1a were designed: QL-GHSR-ful-s1 (10 pmol/μl) and QL-GHSR-ful-as1 (10 pmol/μl) for long-type GHS-R1a (GHS-R1a-L, **acc#AB469019**); QL-GHSR-ful-s2 (10 pmol/μl) and QL-GHSR-ful-as2 (10 pmol/μl) for short-type GHS-R1a (GHS-R1a-S, **acc#AB469019**) (Table 1). RT-PCR for GHS-R1a-L was performed using the cerebellum cDNA as a template by *HotSTAR Taq* Plus mix with reaction conditions at 95˚C for 5 min and subsequent 35 cycles of 95˚C for 0.5 min, 55˚C for 0.5 min and 72˚C for 1 min, and final extension was 72˚C for 3 min. For GHS-R1a-S, RT-PCR was performed by *PrimeSTAR Max* mix using diluted plasmid cloned GHS-R1a-L as the template (TaKaRa) with reaction conditions at 98˚C for 10 sec and subsequent 30 cycles of 98˚C for 10 sec, 57˚C for 20 sec and 72˚C for 10 sec. For GHS-R1b-L **(acc#AB469022)**, RT-PCR was performed with the same condition with GHS-R1a-L **(acc#AB469019)** using QL-GHSR-ful-s1 (10 pmol/μl) and QL-GHSR-ful-as2 (10 pmol/μl) (Table 1). As a result, open reading frames of GHS-R1b-L **(acc#AB469022)** and GHS-R1bV-L **(acc#AB469021)** were obtained. The amplified cDNA was subcloned into the pcDNA3.1-V5-His-TOPO vector after overhanging reaction if necessary. A vector showing correct orientation of the insert for protein expression and the correct GHS-R sequence was subcultured. The plasmid

204 vector was isolated using a HiSpeed Plasmid Midi kit (QIAGEN GmbH) and was
205 diluted to 1 μg/μl for a transfection experiment.
206 Changes in intracellular Ca^{2+} concentrations were measured using $FLIPR^{tetra}$
207 (Molecular Devices, Menlo Park, CA). Human embryonic kidney 293 (HEK293) cells
208 were cultured in DMEM containing 10% fetal calf serum (FCS) at a density of 1×10^6
209 cells in a collagen-coated 10-cm dish for 24 h. An expression vector containing the open
210 reading frame of quail GHS-R1a-L **(acc#AB469019)**, GHS-R1a-S **(acc#AB469019)**,
211 GHS-R1aV-L **(acc#AB469020)**, GHS-R1b-L **(acc#AB469022)** and GHS-R1bV-L
212 **(acc#AB469021)** (2.5 μg) was transfected with FuGENE6 (Roche Diagnostics,
213 Mannheim, Germany) according to the manufacturer's protocol. Twenty-four hours
214 after transfection, the cells were plated onto a poly-D-lysine (Sigma Chemical, St. Louis,
215 MO)-coated 96-well black plate (Corning Inc., Wilker Barre, PA) at a density of 3×10^4
216 cells per well. Twenty hours after plating, cultured medium was aspirated, and 100 μl
217 fluorescent dye solution containing 4.4 μM Fluo-4AM (Invitrogen) and 1% FCS,
218 0.045% pluronic acid (Invitrogen) in a working buffer (1×Hank's BSS [Invitrogen]-20
219 mM HEPES buffer containing 250 μM probenecid [Sigma Chemical]) was loaded into
220 each well. The plate was incubated for 1 h at 37°C in a CO_2 incubator, and the plate was
221 washed three times with a working buffer by an automatic washing machine, followed

by the addition of 100 µl synthetic chicken GRLN 26-C8 [8], quail GRLN (Peptide Institute Inc., Osaka, Japan), GHRP-6 (Bachem AG, Bubendorf, Switzerland) or hexarelin (Phoenix Pharmaceutical Inc., Belmont, CA) at doses of 0.1, 1, 10, 30 and 100 nM in a working buffer containing 0.001% Triton X-100 using the automated FLIPR system. Changes in intracellular Ca^{2+} concentrations were measured by excitation at 488 nm and emission at 500-560 nm.

2.5 Quantitative real-time PCR for chicken GHS-R1a

Quantitative real-time PCR (qPCR) for chicken GHS-R1a **(acc#AB469019)** was performed using a LightCycler System (Roche Applied Science, Mannheim, Germany) and a QuantiTect SYBR Green PCR Kit (QIAGEN GmbH). Total RNA was extracted separately by TRIzol reagent from intact tissues and muscle layer specimens of the esophagus, crop, proventriculus, gizzard, duodenum, jejunum, ileum and rectum of three individuals that had been stored in RNAlater. First-strand DNA was synthesized from 2 µg DNase-I (Invitrogen)-treated total RNA using SuperScript II reverse-transcriptase (Invitrogen) with random primers. The resultant cDNA was cleaned up with a QIAquick PCR Purification Kit (QIAGEN GmbH) for removing factors that interfere with PCR reaction. A primer set (sense: 5'-GGG CCG TCT CCT

240 TCA TTA GTG-3’ and an anti-sense: 5’-TTC CTC TTC CTC CTC CAC AGC-3’) was
241 used. The expected amplicon size was 232 bp. The amplification conditions were 95°C
242 for 15 min, and subsequent 40 cycles at 94°C for 15 sec, 59°C for 30 sec and 72°C for
243 20 sec. The reaction mixture consisted of 1x master mix and 250 nM each of primer and
244 template (80 ng total RNA equivalent). For quantification of GHS-R mRNA copy
245 number, the pCR II-TOPO vector into which a 232-bp chicken GHS-R1a fragment had
246 been cloned was linearized by restriction with *Xba-I*, and serial dilutions of the
247 linearized plasmid from $1x10^6$ to $1x10^3$ were used to generate a linear regression line.
248

249 **2.6. Quantitative real-time PCR for quail GHS-R1a**

250 A QuantiFAST SYBR Green PCR Kit (QIAGEN GmbH) was used for qPCR
251 for quail GHS-R1a. Total RNA was extracted separately by TRIzol reagent from whole
252 tissues and the muscle layers and mucosal layers of the esophagus, crop, proventriculus,
253 gizzard, duodenum, jejunum, ileum, rectum and caecum of four individuals that had
254 been stored in RNAlater. First-strand cDNA was synthesized from 1 μg total RNA using
255 a QuantiTect RT Kit (QIAGEN GmbH). The resultant cDNA was directly used as a
256 template without purification. The primers used were a sense primer (5’-CAG ATC
257 GTG AAG ATG CTA GTT GTG-3’) and an anti-sense primer (5’-GCT GAG GTA GAA

GTG GAC AAA GGA-3'). The expected amplicon size was 168 bp. The amplification conditions were 95°C for 5 min and subsequent 35 cycles at 95°C for 10 sec and 60°C for 30 sec. The reaction mixture consisted of 1x master mix and 250 nM each of primer and template (200 ng total RNA equivalent). For quantification of quail GHS-R1a cDNA copy number, a linear regression line was generated by a serially diluted linearlized quail GHS-R1a fragment (692 bp) obtained by 3'-RACE PCR cloned into the pCRII vector.

2.7. Contraction study for gastrointestinal tracts of the chicken and quail

Smooth muscle strips from different parts of the gastrointestinal tract in the chicken and quail were suspended vertically in an organ bath (5 ml) to measure longitudinal muscle contraction. The organ bath contained warmed (37℃) Krebs solution (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_2$, 25 and glucose, 11.5 equilibrated with 95%O_2 + 5%CO_2 (pH 7.4). Mechanical activity of the preparations was measured with an isometric force transducer (SB-11T, Nihon Kohden, Tokyo, Japan) and recorded on an ink-writing recorder. Initial load was set at 0.5 g for each preparation. The preparations were rinsed with Krebs solution every 15 min and allowed to equilibrate for 1 h. Prior to the addition of GRLN, each strip was

276 subjected to 3 or 4 stimulations with 50 mM KCl until a reproducible contraction was
277 obtained. In order to examine whether GRLN causes contraction of gastrointestinal
278 smooth muscle preparations, chicken GRLN 26-C8 and quail GRLN at 1 μM were
279 applied to an organ bath and the evoked responses were observed as previously
280 described [21]. The amplitude of contractions among preparations was normalized by a
281 standard contraction of 50 mM KCl and expressed as a relative contraction (%).
282 Region-specific amplitudes of contraction by GRLN were compared between the
283 chicken and quail.

284 MTL-induced gastrointestinal muscle contraction was also compared between
285 the chicken and quail. Chicken MTL (custom order in Peptide Institute Inc.) was applied
286 cumulatively to an organ bath at doses of 0.1 nM to 1μM, and concentration-response
287 curves were constructed as previously described [21]. The curves were analyzed by a
288 sigmoid non-linear regression fit using Origin 7.0 (Origin Lab, USA) to determine the
289 molar concentration of the agonist producing 50% (EC_{50}) of its maximal effect (E_{max}).

290

291 **2.8. Peptides**

292 Chicken GRLN 26-C8 was synthesized by Asubio Pharma. Co., Ltd. (Gunma,
293 Japan). Quail GRLN and chicken MTL were synthesized by Peptide Institute Inc.

294 (Osaka, Japan). Their purity was confirmed by a single peak of high-performance liquid
295 chromatography. Rat GRLN and human GRLN were purchased from Peptide Institute
296 Inc. Growth hormone releasing peptide-6 (GHRP-6) and hexarelin were purchased from
297 Bachem Co., Ltd. (Bubendorf, Switzerland) and Phoenix Pharmacol. Inc. (Belmont,
298 CA), respectively.
299

300 **2.9. Statistical analysis**

301 The results are expressed as the means ± S.E.M of more than four experiments.
302 The significance of differences between the values was determined at $P < 0.05$ using
303 paired or unpaired Student's t-test as appropriate for single comparisons or one-way
304 ANOVA followed by Bonferroni Dunnett's t test for multiple comparisons.
305

306 **3. Results**

307 **3.1. Identification of quail GHS-R**

308 We isolated a 1308-bp cDNA encoding a GHS-R-like protein. Two ATG
309 initiation codons for translation were found at positions 54 and 75; the full-length cDNA
310 was composed of a 74- or a 53-bp 5'-untranslated region (UTR), an open reading frame
311 of 1065 or 1044 bp, which encodes a 354- or 347-amino acid protein, and a 190-bp

312 3’-UTR. Comparison of the long-type quail GHS-R-like protein with other GHS-R1a
313 sequences revealed that numerous consensus sequences for GHS-R1a are highly
314 conserved (Fig. 1). Chicken GHS-R1a showed 97% identity with the quail protein at the
315 nucleotide level and 98% identity at the amino acid level. Identity of the amino acid
316 sequence compared with other GHS-Rs was 72% for rat, 72% for zebrafish-1a, 68% for
317 zebrafish-2a, 60% for tilapia, 61% for seabream, 60% for pufferfish, and 60% for
318 rainbow trout. Therefore, quail GHS-R-like proteins were designated as quail GHS-R1a,
319 and its long- and short-type proteins were named GHS-R1a-L and GHS-R1a-S,
320 respectively (Fig. 2, **acc# AB469019**).
321 In the process for identifying the full-length GHS-R1a cDNA, a variant, in
322 which 48-bp nucleotides were deleted at 814-861 of the quail GHS-R1a-L, was also
323 identified. This deduced protein was considered to be an ortholog of chicken
324 GHS-R1aV [11, 12] (Fig. 2, **acc# AB469020**). In 3’-RACE PCR, we obtained two
325 cDNA fragments that have different nucleotide sequences in the 3’-end and encoded
326 different proteins from GHS-R1a: one was a 930-bp product and encoded a
327 309-amino-acid protein, and the other was a 938-bp product and encoded a
328 302-amino-acid protein. The former was considered to be a product in which an 8-bp
329 deletion had occurred from the latter sequence, resulting in a frame shift. These proteins

were considered to be orthologs of GHS-R1b, which has been reported in other animals. We designated these two proteins as quail GHS-R1bV (**acc # AB469021**) and GHS-R1b (**acc # AB469022**), respectively (Fig. 2). In fact, genomic PCR with QL-GHSR-S2 and QL-GHSR-AS2 amplified a 2661-bp partial gene fragment (**acc# AB490327,** Fig. 3), and the two GHS-R1b isoforms were found in the genomic DNA sequence. As shown the nucleotide and amino acid sequences by *green* and by *bold black letters*, respectively, open reading frame of the GHS-R1a sequence is present in the identified gene, and intron sequence, which is shown by *regular black letters* divided the exon. C-terminal GHS-R1b sequence, which is shown in *purple* and *red letters*, was found in the portion extended from 5' splice site of the intron, while C-terminal GHS-R1bV sequence, as shown in *purple* and *blue letters,* was generated by an underlined 8-bp nucleotide deletion and resulted in frame shift (Fig.3).

We also found another variant of GHS-R in the process of qPCR validation. A 268-bp product was highly expressed in the proventriculus and gizzard and contained a 147-bp nucleotide sequence, which has not been found in the GHS-R1a sequence (data not shown). Genomic PCR revealed that complex alternative splicing of the GHS-R gene generated the product (**acc# AB490327**, Fig. 3): the nucleotide sequence is shown by *italics*, the splice and boundary sites are shown by an arrow and #, ##, in which the

identical mark bounds, and deduced amino acid sequence from the generated nucleotide is shown in *orange letters*.

3.2. Functional analyses of quail GHS-R

The two identified quail GHS-R1a cDNAs were transiently expressed in HEK 293 cells, and the cells were treated with chicken GRLN, quail GRLN and two different GHSs, GHRP-6 and hexarelin. Both quail GHS-R1a-L and GHS-R1a-S showed the same degrees of responses to GRLN and GHSs at concentrations of 0.1 nM to 100 nM (Figs. 4A and 4B). Although the maximum responses of the four ligands were almost same, a marked difference was observed in the responsiveness. Chicken GRLN was highly effective to the receptors. However, quail GRLN, GHRP-6, and hexarelin showed similar effectiveness to the receptors (Fig. 4). On the other hand, the cells transfected with quail GHS-R1aV-L, GHS-R1b-L and GHS-R1bV-L did not respond at any doses of reagents tested (data not shown).

3.3. Expression of GHS-R1a mRNA in chicken and quail gastrointestinal tracts

Expression of GHS-R1a mRNA was examined in different regions of the quail gastrointestinal tract, including the esophagus, crop, proventriculus, gizzard, duodenum,

366 jejunum, ileum and colon. Since gastrointestinal mucosa possibly influences the amount
367 of GHS-R1a mRNA, GHS-R1a mRNA expression levels in whole gastrointestinal tract
368 as well as in smooth muscle layers without mucosa were measured. Quail GHS-R1a
369 mRNA expression in intact tissue was highest in the colon, moderate in the esophagus
370 and duodenum, and low in other regions (crop, proventriculus, gizzard, jejunum and
371 ileum) (Fig. 5A). The region-related heterogeneous expression of GHS-R1a mRNA was
372 more marked in the smooth muscle layer preparations. On the other hand, chicken
373 GHS-R1a mRNA expression in whole tissues was highest in the colon, moderate in the
374 esophagus, crop, duodenum and ileum, and low in the proventriculus, gizzard and
375 jejunum (Fig. 5B). The region-dependent heterogeneous expression of GHS-R1a mRNA
376 was also more marked in the smooth muscle layer preparations, and mRNA expression
377 in the colon and esophagus was high. Comparison of the expression levels of GHS-R1a
378 mRNA in the corresponding gastrointestinal regions of both avian species revealed a
379 significant correlation in expression patterns of GHS-R1a mRNA in the quail and
380 chicken ($R=0.92$, $P=0.001$).
381
382 **3.4. Effects of GRLN on the contraction of different regions of chicken and quail**
383 **gastrointestinal tracts**

384 Mechanical actions of the contractility of non-stimulated gastrointestinal
385 muscle strips were examined. Chicken GRLN (1 μM) caused contraction of chicken
386 gastrointestinal smooth muscle strips in a region-specific manner as previously
387 described [21] (Fig. 6, upper). The contractile responses were strongest in the crop (70.5
388 ± 5.3%, n=14) and colon (69.7 ± 7%, n=5), moderate in the proventriculus (32.7 ± 4.3%,
389 n=9), and weak in the small intestine (duodenum: 5.9 ± 1.1%, n=5; jejunum: 19.0 ±
390 2.7% n=5; ileum: 10.8 ± 2.0%, n=5) (Fig. 7). There was a significant positive
391 correlation between the GRLN-induced contraction and GHS-R1a mRNA expression in
392 different regions of the chicken gastrointestinal tract (R=0.91, P=0.005).
393 On the other hand, the contractile responses to quail GRLN (1 μM) in the quail
394 gastrointestinal tract (especially, crop, proventriculus and colon) was small compared to
395 the responses to chicken GRLN in the chicken gastrointestinal tract (Figs. 6 and 7). The
396 relative amplitudes of contraction in the crop, proventriculus, duodenum, jejunum,
397 ileum and colon were 6.9 ± 0.5% (n=5), 7.6 ± 0.7% (n=9), 2.4 ± 2.4% (n=4), 3.1 ± 2.6%
398 (n=4), 2.7 ± 0.7% (n=8) and 11.5 ± 2.5% (n=7), respectively (Fig. 7). Although a
399 significant correlation was not found between the contractile responses and quail
400 GHS-R1a mRNA expression (R=0.68, P=0.14), the contractile responses to GRLN in

401 the crop, proventriculus and colon were slightly larger than the responses in the small
402 intestine, as has been observed in the chicken gastrointestinal tract.

403 To confirm contractile activity of quail GRLN, the response to quail GRLN (1
404 μM) was also investigated in the chicken crop. The amplitude of contraction induced by
405 quail GRLN (1 μM, 57.4 ± 9.7%, n=5) was comparable with that by chicken GRLN (64
406 ± 12%, n=5). Rat GRLN and human GRLN (1 μM) were significantly less effective in
407 producing muscle contraction of the chicken crop (14 ± 3.7%, n=4 for rat GRLN, 12 ±
408 2.3%, n=4 for human GRLN). In the chicken proventriculus, quail GRLN (31 ± 6.5%,
409 n=6) and chicken GRLN (27 ± 6%, n=6) caused similar degrees of contraction. In
410 contrast, the responses of the quail gastrointestinal tract to chicken GRLN were also
411 small, as was observed in the case of quail GRLN (crop: 10 ± 1.7%, n=4;
412 proventiculus :7.2 ± 1.1, n=6; colon: 8.5 ± 1.5, n=4; duodenum: 3.2 ± 1.3, n=3;
413 jejunum: 2.6 ± 1.0%, n=4 and ileum: 3.6 ± 0.8%, n=7).

414

415 **3.5. Effects of chicken MTL in chicken and quail gastrointestinal tracts**

416 Chicken MTL (0.1 nM -1 μM) caused region-specific contractions in the
417 chicken gastrointestinal tract (Fig. 8). The contractile response was strong in the small
418 intestine (duodenum, jejunum and ileum, E_{max} = approximately 110%, EC_{50} = 4-6 nM),

419 moderate in the proventriculus (E_{max} = 35%, EC_{50} = 30 nM) and colon (E_{max} = 33%,
420 EC_{50} = 26 nM), and weak in the crop (E_{max} = 17%, EC_{50} was not determined). Chicken
421 MTL (0.1 nM -1 μM) also caused contraction of the quail small intestine with a degree
422 of magnitude similar to that observed in the chicken (EC_{50} = 2-4 nM). The ranking order
423 of E_{max} was jejunum (83%) = duodenum (80%) > ileum (68%) > colon (42%) > crop
424 (28%) > proventriculus (17%) similar to the case of the chicken (Fig. 8).
425

426 **4. DISCUSSION**

427 In the present study, we identified GHS-R in the Japanese quail and examined
428 the extent of GHS-R1a mRNA distribution in Japanese quail gastrointestinal tract and
429 the effect of GRLN on the contractility of gastrointestinal tract. GHS-R1a expression
430 and GRLN-induced response were also investigated in the chicken and compared with
431 quail. Region-specific action of GRLN and region-related heterogeneous expression of
432 GHS-R1a mRNA in the gastrointestinal tract were demonstrated to be conserved in two
433 avian species, although there was marked difference in contractile efficacy (chicken >
434 quail). In addition, it is interesting that region-specific actions were observed for two
435 similar gut peptide hormones, GRLN and MTL, which have never seen in rodents
436 because they do not have endogenous MTL and MTL receptors [25, 26].

437 We isolated cDNA encoding a 354- or 347-amino-acid protein with numerous
438 consensus sequences to other GHS-R1a. Since the identified proteins showed higher
439 identity to zebrafish GHS-R1a than to zebrafish GHS-R2a, we designated these proteins
440 quail GHS-R1a, while the long-type and short-type receptors were named GHS-R1a-L
441 and GHS-R1a-S, respectively. It is convinced that these quail GHS-Rs show the highest
442 identity to chicken GHS-R1a [11, 12]. In the chicken, a protein homologous to quail
443 GHS-R1a-L is not translated from the cDNA because a frame shift occurs by an
444 insertion of one nucleotide in the chicken. Therefore, quail GHS-R1a-S would be an
445 ortholog of chicken GHS-R1a. Indeed, functional analysis has demonstrated that both
446 quail GHS-R1a-L and 1a-S act as functional receptors for quail GRLN in the Japanese
447 quail. At first it was assumed that quail GRLN was more effective to quail GHS-R than
448 was chicken GRLN, but quail GRLN was less sensitive than chicken GRLN in this
449 experiment. Since amino acid sequence of quail GRLN and chicken GRLN was
450 different in three positions (8, 17, 22) [6, 10], these differences affect the binding with
451 the quail GHS-R and the subsequent Ca^{2+} responses by GRLNs. Although physiological
452 meaning of different responsiveness of chicken and quail GRLNs is not clear, it might
453 be suggested that quail is a low sensitive avian species to GRLN compared with
454 chicken.

455 We identified four splice variants of GHS-R, named GHS-R1aV, GHS-R1b,
456 GHS-R1bV and GHS-R1tv-like receptor. Chicken GHS-R1aV (or GHS-R1c termed by
457 Geelissen et al. [11]) is an alternative spliced variant of GHS-R1a that lacks
458 transmembrane domain-6 by deletion of 48-bp nucleotides [12]. In quail GHS-R1aV,
459 the same numbers of nucleotide have been deleted. There has not been any reports on
460 the function of GHS-R1aV, even in the chicken; but the present study showed for the
461 first time that GRLN or two GHSs do not increase intracellular Ca^{2+} concentrations in
462 HEK 293 cells expressing quail GHS-R1aV, suggesting that GHS-R1aV is not involved
463 in GRLN signalling.

464 GHS-R1b is another splice variant that is known to be present in mammals and
465 fish, and it contains a part of the intron sequence of the GHS-R gene at the amino acid
466 sequence of the C-terminus [27, 28]. In this study, we identified for the first time two
467 GHS-R1b cDNAs encoding a 302- or 309-amino-acid protein in birds, which is
468 structurally different from GHS-R1aV. The two proteins were designated GHS-R1b and
469 1bV, respectively. Genomic PCR for quail GHS-R revealed the presence of those
470 nucleotide sequences, and GHS-R1bV is generated by a frame shift after an 8-bp
471 deletion. Although a similar GHS-R1b sequence is present in the chicken GHS-R gene,
472 it has not been yet determined whether the cDNA is generated [11, 12]. In this study, an

473 increase in intracellular Ca^{2+} concentrations was not elicited in GHS-R1b-L or
474 GHS-R1bV-L expressing cells by GRLN or GHSs. Little is known about the function of
475 GHS-R1b in vertebrates, but it has been demonstrated that expression, translocation and
476 activity of GHS-R1a are modulated by the protein [28-30].

477 Furthermore, as previously reported [13], we identified a cDNA when
478 validating real-time PCR that is considered as GHS-R1tv. We named the protein
479 GHS-R1tv-like receptor. Genomic PCR revealed that the cDNA is generated by
480 complex splicing of the GHS-R gene. In the present experiment, we predominantly
481 detected the cDNA in the proventriculus and gizzard, but we did not examine its
482 distribution of other tissues including gonad as shown in Sirotkin et al. [13]. Little is
483 known about the function of GHS-R1tv in birds.

484 GRLN is synthesized and stored in the gastric mucosa cells and affects
485 gastrointestinal motility [2]. However, expression of GHS-R1a mRNA in the
486 gastrointestinal tract has only been investigated in humans thus far, and homogeneous
487 expression along the digestive tract has been reported [31]. In this study, GHS-R1a
488 mRNA was found to be heterogeneously expressed in the gastrointestinal tracts of the
489 quail and chicken, and the expression patterns were similar in the two avian species,
490 i.e., highest in the colon, moderate in the esophagus and crop, and low in the

proventriculus and small intestine. GHS-R1a mRNA expression was higher in muscle layer preparations than in whole gastrointestinal preparations with mucosa, and the expression was negligible in mucosa preparations, suggesting that GHS-R1a is mainly located at smooth muscle layers including enteric nerves. This supports a previous observation of myogenic and neurogenic contractile mechanisms of GRLN in the chicken gastrointestinal tract [21]. These results suggest that GRLN acts as a gut hormone to regulate contractility of the crop and colon in avian species.

GRLN-induced contraction was considerably weak in the quail compared with that observed in the chicken gastrointestinal tract, despite the fact that similar amounts of GHS-R1a mRNA were expressed. Similar discrepancy between GHS-R mRNA expression level and contractile function has been reported in humans: GHS-R mRNA and protein are expressed in the stomach and colon [31], but GRLN is ineffective in causing mechanical responses and in modifying neural contraction [32]. Although the underlying mechanisms are not known at present, the following speculations may account for the discrepant mechanical responses to GRLN in the chicken and quail: 1) GHS-R1a mRNA is not translated in GHS-R1a protein in the quail, 2) most of GHS-R1a in the quail are not functional for eliciting the contractile responses, and 3) distribution of GHS-R1a in smooth muscles and neural components differs in the

509 chicken and quail. It was firstly assumed that GRLN stimulates gastrointestinal
510 motility more potently in the quail than that observed in the chicken, because strong
511 gastrointestinal contraction would be needed in the quail after increase of food intake
512 by peripheral injection of GRLN [19]. However, the results obtained in the present
513 study were opposite. To obtain a better understanding of the functional relevance of
514 GRLN for food intake and gastrointestinal motility, the effects of GRLN in other bird
515 species must be examined.

516 Motilin is a GRLN-related peptide, and it has been shown that the MTL
517 receptor and GHS-R would have been derived from a common ancestral gene [23, 24].
518 Chicken MTL receptor was highly expressed in the proventriculus and duodenum [24],
519 although homogenous expression of MTL receptor mRNA in the human
520 gastrointestinal tract has been reported [31]. In the present study, chicken
521 MTL-induced contraction was strong in the small intestine including the duodenum,
522 jejunum and ileum, moderate in the proventriculus and colon, and weak in the crop of
523 both the quail and chicken. This is the first report on a region-specific common
524 contractile response of MTL in gastrointestinal tract of avian species. In the chicken,
525 MTL has been suggested to be a mediator of rhythmic oscillating complexes of the
526 small intestine [33]. It is notable that GRLN and a related peptide, MTL, separately

527 regulate gastrointestinal motility in a region-specific manner in these birds: GRLN
528 regulates motility of the upper (crop) and lower (colon) guts, and MTL regulates
529 motility of the middle gut (small intestine). A recent immunohistochemical study
530 demonstrated that GRLN and MTL are simultaneously secreted from a prominent
531 endocrine cell population in the human small intestine [34]. Therefore, it is highly
532 possible that GRLN and MTL orchestrate gastrointestinal motility in the chicken and
533 quail, like in humans. An avian species, especially the chicken, is a good animal model
534 to examine the interaction of these two peptides, because rodents (rats and mice),
535 MTL-deficient species [25, 26], are insensitive to MTL.

536 In summary, we identified several types of GHS-R in the Japanese quail.
537 Distribution of GHS-R1a mRNA in the gastrointestinal tract coincided well with the
538 muscle contraction properties, which are strong in the esophagus and colon, in both the
539 quail and chicken. However, the contractile responses to GRLN were weak in the quail
540 compared to those in the chicken even though peripheral exogenous GRLN stimulates
541 food intake in the quail. It is necessary to examine the relationship between food intake
542 and gastrointestinal motility induced by GRLN. MTL stimulated muscle contraction in
543 small intestinal regions including the duodenum, jejunum and ileum, in which the effect
544 of GRLN is weak. These region–specific effects of GRLN and MTL are unique in avian

545 species, and different contractile responses in the chicken and quail reflect species
546 difference in the regulation of gastrointestinal motility by GRLN.
547
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Table 1. Primers used in this experiment

GHS-R-dSES1 : AAY YTY TAY CTS TSY AGY ATG GC
GHS-R-dSES2 : GAY CTS CTS ATY TTY CTS TGY ATG CC
GHS-R-dANT1 : TTR ATS GCN GCR CTS AGR TAR AA
QL-GHSR-S1 : TCG AGC ACG AGA ACG GCA CCA ACC
QL-GHSR-S2 : GGT GTG GAT CTC CAG CAT CTT CTT
QL-GHSR-S3 : GGC ACC AAC CCG CTG AGC ACC AAC
QL-GHSR-S4 : ATC TTC TTT TTC TTG CCT GTC TTC
QL-GHSR-AS1 : GCT GAG GTA GAA GTG GAC AAA GGA
QL-GHSR-AS2 : AGG CAA CCA GCA GAG TAT GAA AGC
QL-GHSR-AS3 : TTA ATC CAA GTG TTG ATT GCT ACC
QL-GHSR-AS4 : CAC AAC TAA AAC AAA AAA CAG CAG
QL-GHSR-ful-s1 : ATG CGC AGC CGC AGC GGC ACG ATG
QL-GHSR-ful-s2 : ATG CGG GAG GGG AGC GCG GAG AAC
QL-GHSR-ful-as1 : TCA TGT GGC GAC GGT GGG TTC TGT
QL-GHSR-ful-as2 : TCA AAG GAA AAG GAA GAG TTG TTC

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692 **Figure legends**

693

694 **Fig. 1. Multiple amino acid sequence comparison of GHS-R1a and identified quail**
695 **GHS-R1a-like protein.** *Asterisks* indicate identical amino acids across all species. *Dots*
696 indicate more than half of identical amino acids across all species. Amino acid
697 sequences are available from the DDBJ/EMBL/GenBank databases: quail (**AB469019**),
698 chicken (**AB095995**), rat (**U94321**), zebrafish1a (**XM001335981**), zebrafish2a
699 (**XM001340372**), tilapia (**AB361053**), seabream (**AY151040**), pufferfish (**AF082209**)
700 and rainbow trout (DQTA/LN, **AB362479** and ERAT/IS, **AB362480**),

701

702 **Fig. 2. Deduced amino acid sequences of identified quail GHS-R isoforms**. *Asterisks*
703 indicate identical amino acids across all species. *Dots* indicate more than half of
704 identical amino acids across all species. *Italic letters* indicate amino acid sequence
705 originated from the intron. *Italic bold* letters show different part of sequence from
706 GHS-R1b-L. The nucleotide sequence was deposited in the DDBJ/EMBL/GenBank™
707 databases with the accession numbers **AB469019** for 1a-L and 1a-S, **AB469020** for
708 1aV-L, **AB469021** for 1bV-L and **AB469022** for 1b-L.

709

Fig. 3. Partial nucleotide sequence and deduced amino acid sequence of quail GHS-R gene including an intron. Genomic PCR was performed with QL-GHSR-S2 and QL-GHSR-AS2, and a 2661-bp fragment was obtained. Represented nucleotide number is consecutive from the cDNA (**AB469019**). The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank™ databases with the accession number **AB490327.** Detail of this figure was indicated in the Results.

Fig. 4. Functional analysis of quail GHS-R1a.

Changes in intracellular Ca^{2+} concentrations were examined in human embryonic kidney (HEK) 293 cells that were transfected with quail GHS-R1a-L (A) and 1a-S (B). These cells were treated with chicken ghrelin (●), quail ghrelin (■), GHRP-6 (▲) and hexarelin (▼) at concentrations of 0.1, 1, 10, 30 and 100 nM. Values represent the means ± SEM of triplicate examinations.

Fig. 5. Comparison of expression of GHS-R1a mRNA in gastrointestinal tracts of the quail (A) and chicken (B). Expression of GHS-R1a mRNA in esophagus, crop, proventriculus, gizzard, duodenum, jejunum, ileum and colon strips with mucosa (■, whole preparations) or without mucosa (□, mucosa-free) were analyzed using real-time

qRT-PCR. Each column represents the mean ± SEM of triplicate examinations.

Fig. 6. Representative contractile responses of gastrointestinal preparations to GRLN. Crop, proventriculus, duodenum, jejunum, ileum and colon strips isolated from the chicken and quail were used for the study. Effects of 50 mM KCl (50 K, ▲) and chicken GRLN (1 μM, ●) or quail GRLN (1μM, ●) were examined in the chicken preparations (upper) and quail preparations (lower).

Fig. 7. Comparison of GRLN-induced contractions in the chicken and quail. Mechanical responses to chicken GRLN (1 μM) and quail GRLN (1 μM) in different regions of the chicken (■) and quail (□) gastrointestinal tracts were normalized by the contraction induced by 50 mM KCl, and the relative contractions are represented. GRLN-induced contraction was more conspicuous in the chicken preparations. Each column represents the mean ± SEM of more than 4 examinations.

Fig. 8. Comparison of maximum responses of MTL-induced contraction in the gastrointestinal tracts of the chicken and quail. Chicken MTL was applied to different regions of chicken (■) and quail (□) gastrointestinal tracts cumulatively, and

746 the maximum contraction was normalized by the contraction induced by 50 mM KCl.

747 Each column represents the means ±SEM of more than 4 examinations.

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Figure 1

Fig.1

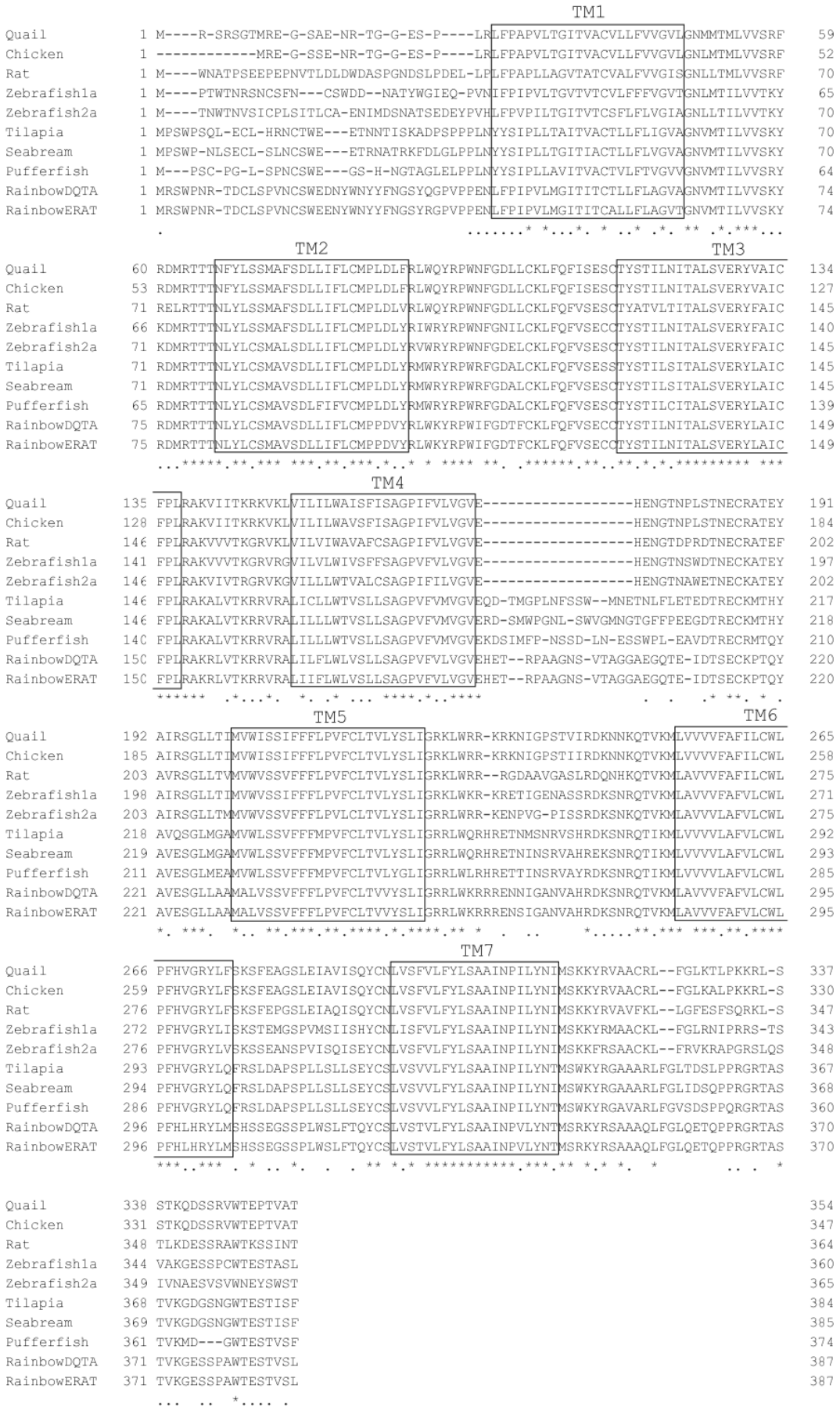

Figure 2

Fig.2

Figure 3

Fig.3

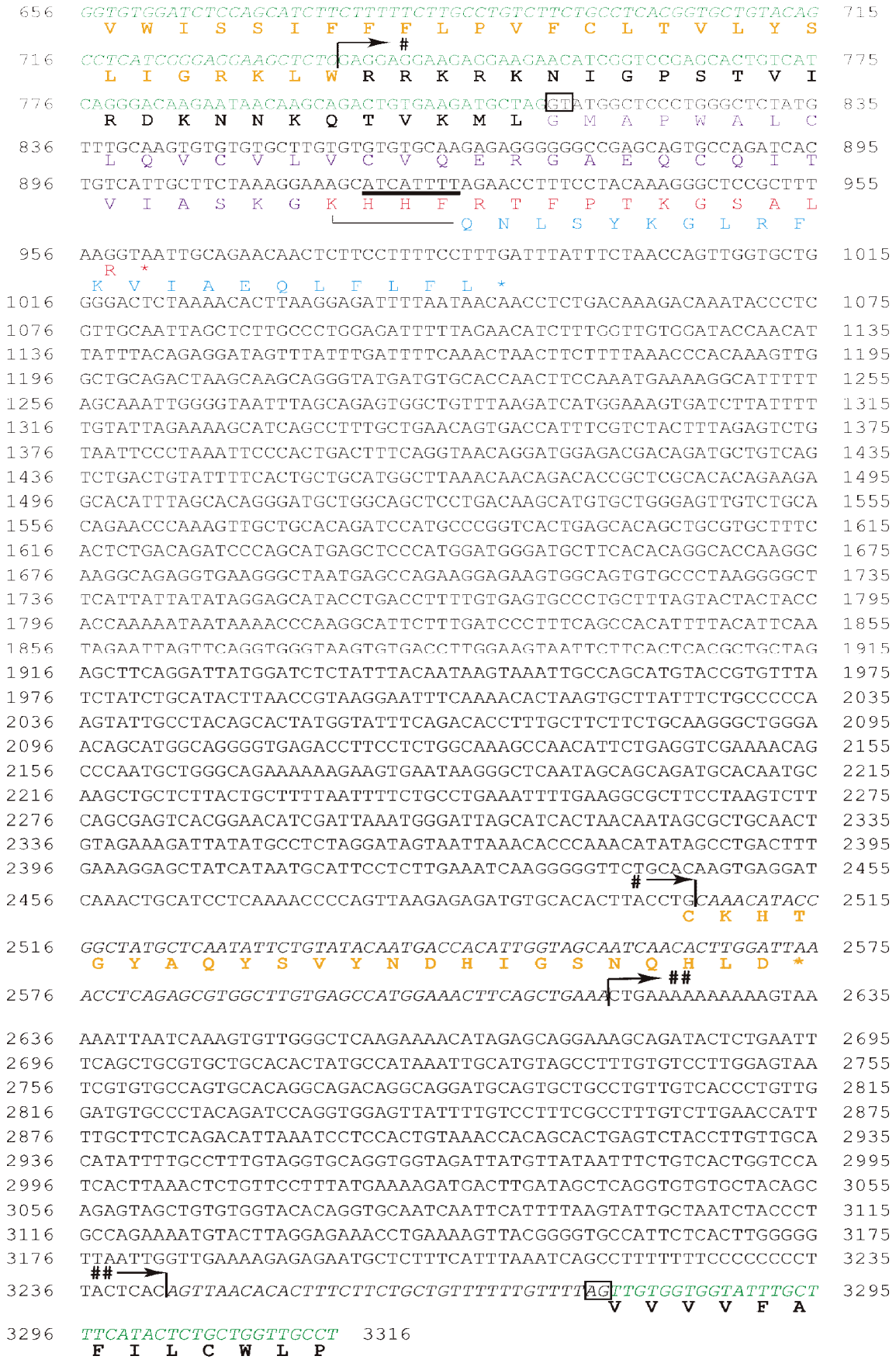

Figure 4

Fig 4

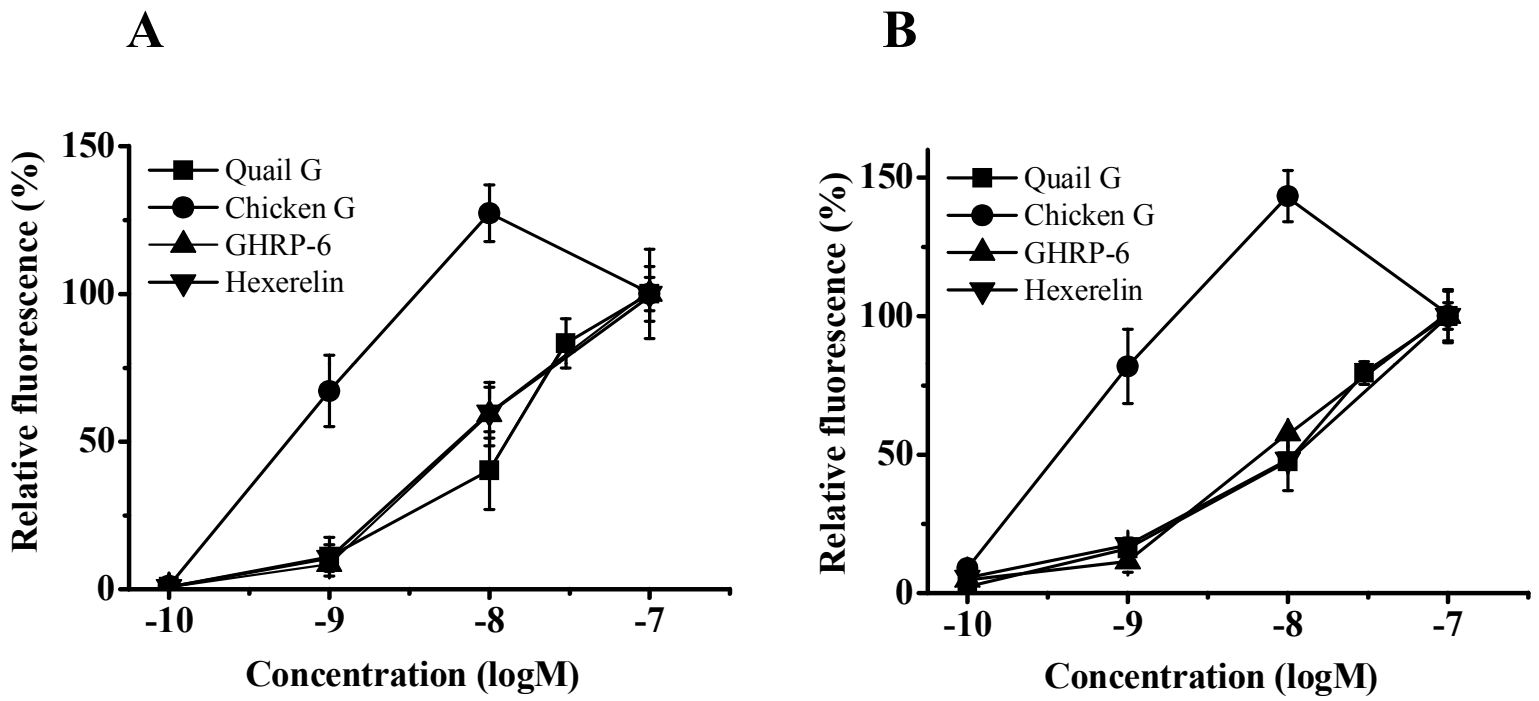

Figure 5

Fig.5

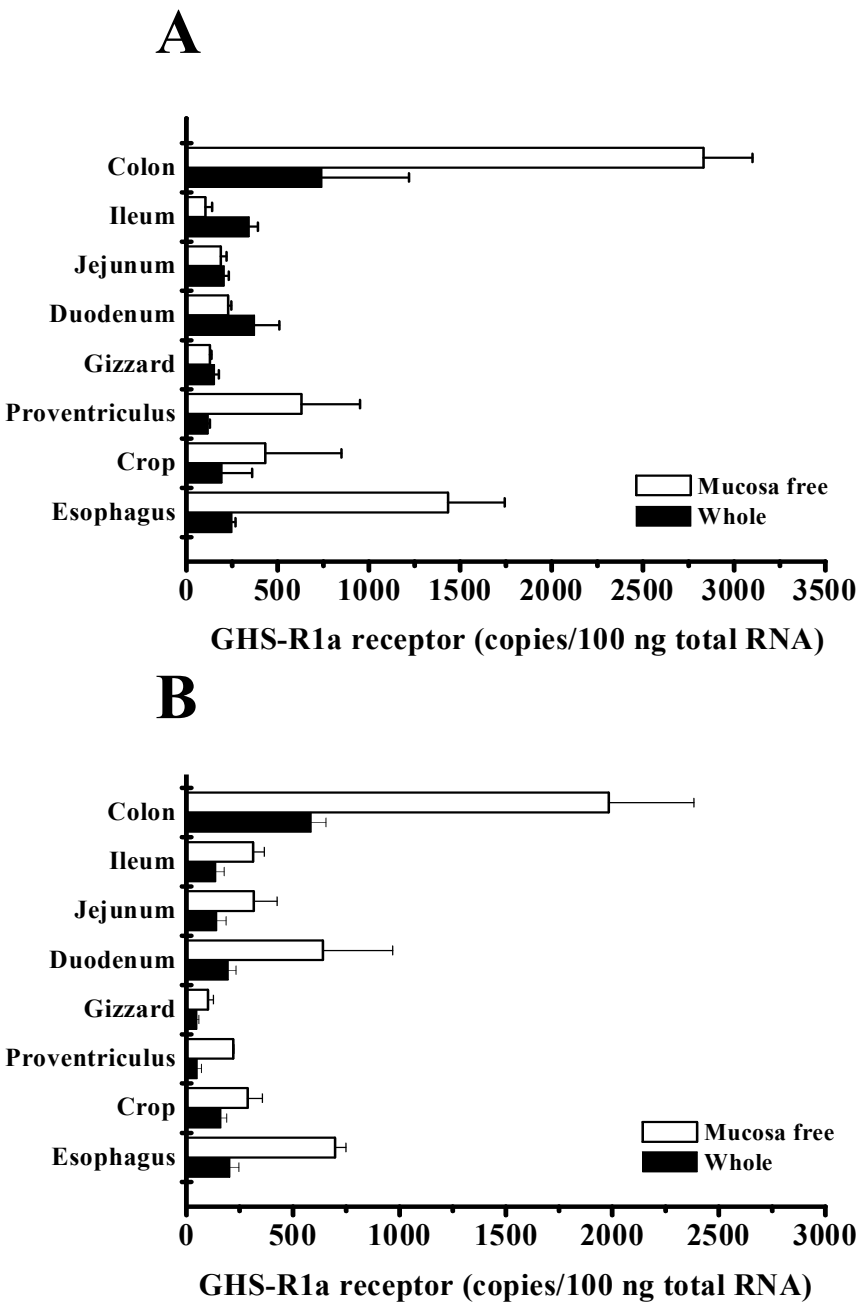

Figure 6

Fig.6

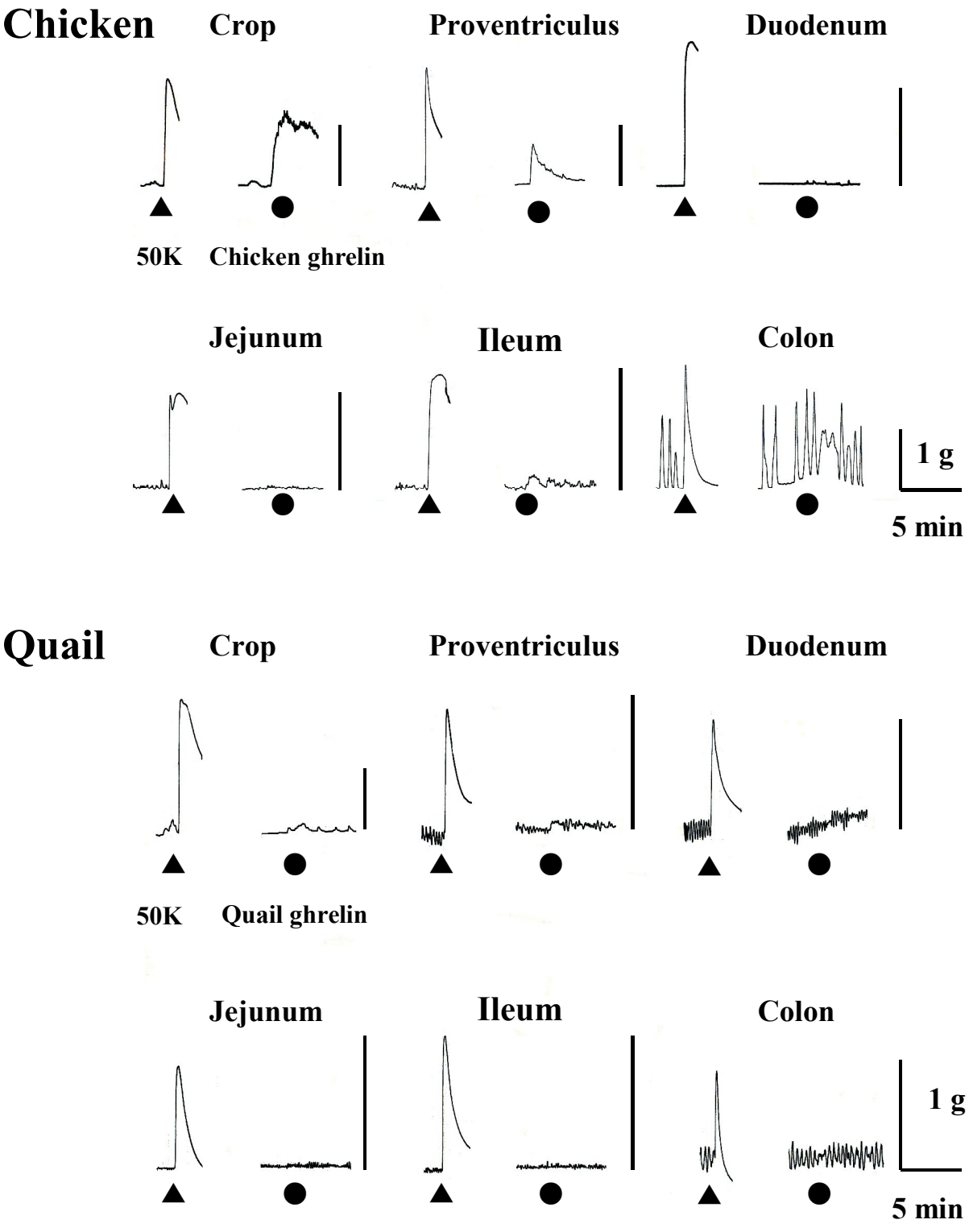

Figure 7

Fig.7

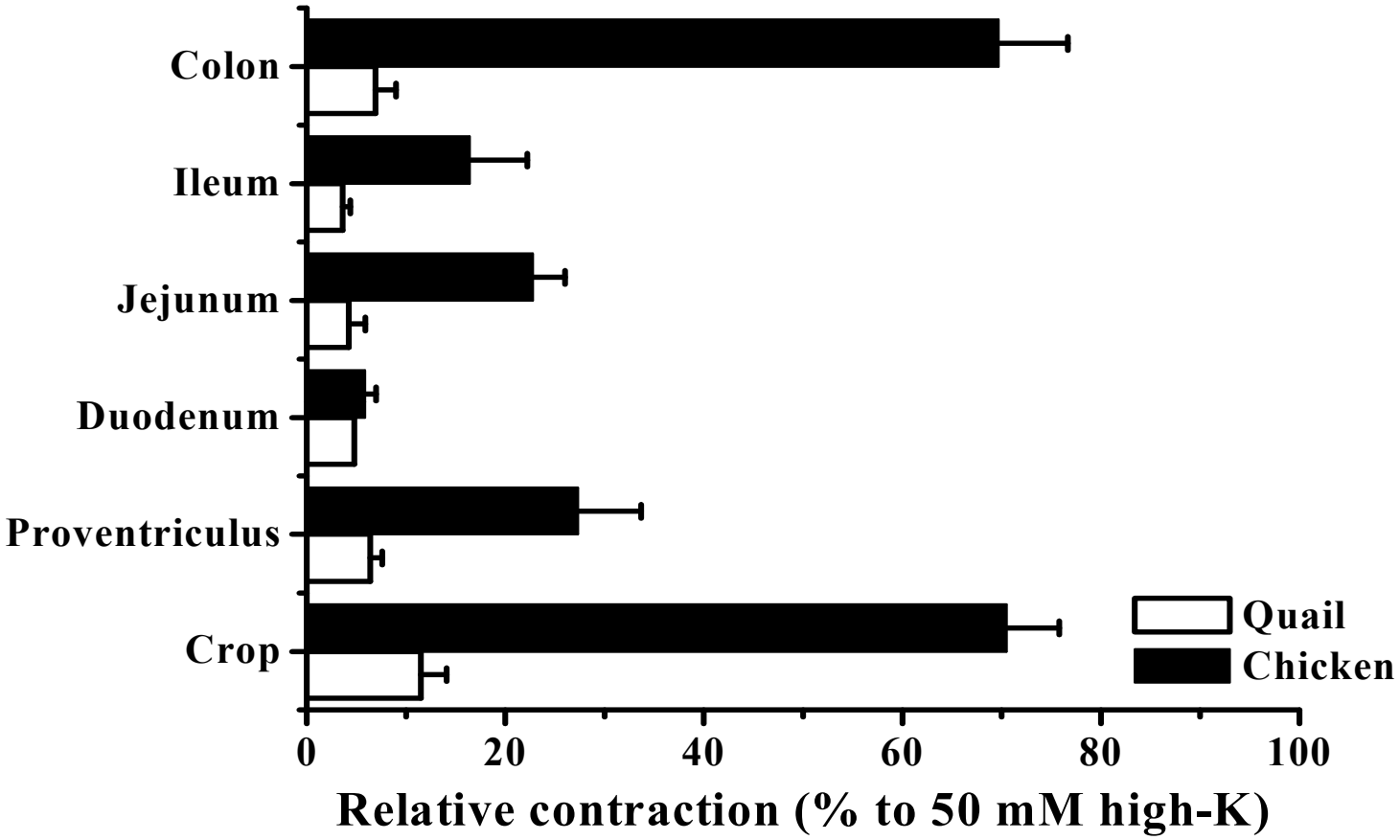

Figure 8

Fig.8