Identification of pheasant ghrelin and motilin and their actions on contractility of the isolated gastrointestinal tract

Shuangyi Zhang[1,2], Yuji Okuhara[2], Mio Iijima[3], Shota Takemi[3], Ichiro Sakata[3], Hiroyuki Kaiya[4], Hiroki Teraoka[2] and Takio Kitazawa[1*]

[1]Department of Veterinary Science, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan.

[2]School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan.

[3]Area of Regulatory Biology, Division of Life Science, Graduate School of Science and Engineering, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338-8570, Japan

[4]Department of Biochemistry, National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan.

*Corresponding author: Takio Kitazawa, Ph.D., Department of Veterinary Science, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan.

Tel: +81-11-388-4795, Fax: +81-11-387-5890

e-mail: tko-kita@rakuno.ac.jp

Abstract

Motilin and ghrelin were identified in the pheasant by molecular cloning, and the actions of both peptides on the contractility of GI strips were examined *in vitro*. Molecular cloning indicated that the deduced amino acid sequences of the pheasant motilin and ghrelin were a 22-amino acid peptide, FVPFFTQSDIQKMQEKERIKGQ, and a 26-amino acid peptide, GSSFLSPAYKNIQQQKDTRKPTGRLH, respectively. **In *in vitro* studies using pheasant GI strips, chicken motilin caused contraction of the proventriculus and small intestine, whereas the crop and colon were insensitive. Human motilin, but not erythromycin, caused contraction of small intestine. Chicken motilin-induced contractions in the proventriculus and ileum were not inhibited by a mammalian motilin receptor antagonist, GM109. Neither atropine (a cholinergic receptor antagonist) nor tetrodotoxin (a neuron blocker) inhibited the responses of chicken motilin in the ileum but both drugs decreased the responses to motilin in the proventriculus, suggesting that the contractile mechanisms of motilin in the proventriculus was neurogenic, different from that of the small intestine (myogenic). On the other hand, chicken and quail ghrelin did not cause contraction in any regions of GI tract. Since interaction of ghrelin and motilin has been reported in the house shrew (Mondal et al., 2012), interaction of two peptides was examined. The chicken motilin-induced contractions were not modified by ghrelin, and ghrelin also did not cause contraction under the presence of motilin, suggesting the absence of interaction in both peptides. In conclusion, both the motilin system and ghrelin system are present in the pheasant. Regulation of GI motility by motilin might be common in avian species. However, absence of ghrelin actions in any GI regions suggests the avian species-related difference in**

regulation of GI contractility by ghrelin.

Key words: Ghrelin, Motilin, Pheasant, Contraction, Small intestine, Proventriculus.

1. Introduction

Motilin, a 22-amino-acid peptide, was discovered from the mucosa of the porcine intestine (Brown et al., 1971, 1973) and it was shown to stimulate gastrointestinal (GI) motility in several mammals through activation of the motilin receptor (GPR38, Feighner et al., 1999) located on enteric neurons and smooth muscle cells (Kitazawa et al., 1994; Broad et al., 2012). In humans, dogs and the house musk shrew (*Suncus murinus*), motilin is thought to be an endogenous regulator of phase-III activity of migrating motor complex (MMC) in the stomach (Itoh et al., 1976; Vantrappen et al., 1979; Sakahara et al., 2010; Mondal et al., 2012). Evidence showing that exogenous motilin causes gastric contractions similar to phase-III contractions and that peaks of endogenous motilin levels are closely associated with gastric phase-III contractions supports the involvement of motilin in induction of the phase-III pattern of MMC. In addition, results showing that the occurrence of gastric phase-III contractions was disrupted by anti-motilin serum and a motilin receptor antagonist supported the notion that motilin is an endogenous mediator of phase-III activity of gastric MMC (Itoh et al., 1976, 1978; Peeters et al., 1980; Lee et al., 1983; Ozaki et al., 2009; Mondal et al., 2012; Ogawa et al., 2012). Rodentia such as mice and rats lack motilin and the motilin receptor (motilin system) (He et al., 2010; Sanger et al., 2011), though motilin system has been present in various mammals (Itoh, 1997; Kitazawa and Kaiya, 2019). Presence of the motilin system has been reported in some birds (chickens and quails) (Kitazawa et al., 1997; Yamamoto et al., 2008; Apu et al., 2016) but not investigated extensively in reptiles, amphibians and in fish.

Ghrelin, a natural ligand for growth hormone secretagogue-receptor 1a (GHS-R1a), has been identified in the gastric mucosa of mammals and non-mammals and has been

shown to be a gut peptide with multiple functions including regulation of GH release, glucose homeostasis, and food intake, endocrine and exocrine pancreatic functions, cardiac function and regulation of GI motility (Kojima et al., 1999; Kojima and Kangawa, 2005; Kaiya et al., 2008; Sato et al., 2012). The multiple functional roles of ghrelin are supported by biochemical evidence that ligand binding sites (GHS-R1a) and *GHS-R1a* mRNA are ubiquitously distributed in the brain and in several peripheral tissues (Gnanapavan et al., 2002; Davenport et al., 2005). Since ghrelin and GHS-R1a show some structural homology with motilin and its receptor and are thought to be derived from the same ancestor gene (Asakawa et al., 2001; Peeters, 2005), the stimulatory action of ghrelin on GI motility has been investigated in humans and some experimental animals including rodents (Fujino et al., 2003; Depoortere et al., 2005; Kitazawa et al., 2005; Tack et al., 2006). **In mice and rats, the ghrelin system is thought as a regulator of gastric MMC observed in the fasting periods** (Fujino et al., 2003, Ariga et al., 2007; Zheng et al., 2009).

The mechanisms by which ghrelin induces GI stimulating action are depending on the species, experimental conditions (*in vitro* or *in vivo*) and GI regions. *In vivo* experiments in conscious rats and *Suncus* showed that ghrelin-induced gastric contraction is partially decreased by vagotomy, suggesting a vago-vagal reflex pathway, and that enteric neurons mediate the ghrelin-induced actions (Fujino et al., 2003; Miyano et al., 2013). **Presence of the GHS-R1a in vagal afferent nerve terminals (Sakata et al., 2003) and enteric neurons (myenteric plexus) has been demonstrated in rats (Dass et al., 2003b).** *In vitro* experiments showed that ghrelin alone did not cause any **contractile** responses in non-electrically stimulated preparations but potentiated electrical stimulation-induced contraction through activation of enteric

121 neural GHS-R1a (Depoortere et al., 2005; Kitazawa et al., 2005). In contrast, chicken
122 ghrelin caused contraction of the chicken crop through activation of smooth muscle
123 receptors (Kitazawa et al., 2007). Smooth muscle GHS-R1a has only been found in
124 chickens, suggesting that chickens are suitable animals for analysis of ghrelin actions in
125 GI motility (Kitazawa et al., 2017a). However, ghrelin did not cause any contraction **in**
126 the GI tract of Japanese quails despite clear expression of *GHS-R1a* mRNA (Kitazawa
127 et al., 2009; Apu et al., 2016). **These contrastive** actions of ghrelin **on the contractility**
128 **of** the chicken and Japanese quail GI tract prompted us to examine the effects of ghrelin
129 on GI **contractility** in other avian species to determine **which the general actions of**
130 **ghrelin (stimulation or no effect) on contractility of avian GI tract are.**
131 Since some mammals, such as dogs and the *Suncus*, expressing both ghrelin and
132 motilin and their receptors in the GI tract, interaction of the two peptides in GI motility
133 has been examined. Ghrelin caused gastric contraction in the presence of a low
134 concentration of motilin in the *Suncus* both in *in vitro* and *in vivo* (Mondal et al., 2012),
135 but ghrelin inhibited the motilin-induced **MMC** in conscious dogs (Ogawa et al., 2012).
136 Expression of both ghrelin and motilin has also been demonstrated in avian species
137 (chickens and quails). There was no interaction of ghrelin and motilin in the quail
138 intestine (Apu et al., 2016). However, study of ghrelin and motilin interaction in GI
139 **contractility** has been limited to the quail, and a comparative study using another avian
140 species is necessary **to determine the interaction of motilin and ghrelin in avian GI**
141 **contractility.**
142 In the present study, we used pheasants (*Phasianus colchicus versicolor*) as another
143 avian species because they are included in *Galliformes* as are chickens and quails and **it**
144 **is possible to compare the actions of ghrelin among closely related species. In**

addition, it was easy to take them from a nearby farm. We first identified the primary structures of motilin and ghrelin in the pheasant by molecular cloning and then examined the mechanical effects of motilin and ghrelin and their interaction in isolated GI strips of the pheasant.

2. Materials and methods

All experiments were performed in accordance with Institutional Guidelines for Animal Care at Rakuno Gakuen University (VH18D1), Ebetsu, Hokkaido, Japan.

2.1. Animals and tissue preparations

Male and female pheasants (*Phasianus colchicus versicolor*, 30-60 days after hatching, 300-450 g, **n=20**) were obtained from a farm in Iwamizawa City, Hokkaido, Japan. The pheasants were anaesthetized with isoflurane, stunned, and bled to death. The crop, proventriculus, small intestine and colon were removed after a midline incision, and their luminal contents were flushed out using ice-cold Krebs solution (**mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25 and glucose, 11.5.** The crop and proventriculus were cut open, and smooth muscle strips in the longitudinal muscle direction (1 mm in width and **10-15** mm in length) were prepared for the contraction study. In the case of a tube-like intestine (duodenum, jejunum, ileum and colon), each intestine was cut into strips of **10-15** mm in length and contraction of the preparations in the longitudinal muscle direction was assessed.

2.2. Cloning of pheasant motilin

Total RNA was extracted from the duodenum of a pheasant by ISOGEN (Nippon

169 Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Trace DNA
170 contamination was removed by DNase digestion (Promega, Madison, WI, USA) and
171 cDNA was synthesized from 2 µg of DNase-treated total RNA using Prime Script II
172 Reverse Transcriptase (Takara Bio, Shiga, Japan) and Oligo-dT with an anchor primer,
173 5'-
174 CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTT
175 TTVN-3'. Primary 3'-RACE PCR amplification was performed with 1 µl of a template,
176 100 pmol/µl of primers for a sense 5'-CCGGTTTGCTCCTGGTGTA -3'and antisense
177 5'-CCAGTGAGCAGAGTGACG -3', and ExTaq DNA polymerase (TaKaRa Bio,
178 Shiga, Japan). The reaction conditions were 94˚C for 2 min followed by 40 cycles of
179 94˚C for 0.5 min, 55˚C for 0.5 min and 72˚C for 0.5 min with final extension at 72˚C
180 for 5min. The resultant product was subjected to second-round nested PCR. Nested
181 PCR was conducted with 1 µl of diluted primary PCR product, 100 pmol/µl of a sense
182 primer 5'- TCAAAGGGCAGAAGAAATCC -3', antisense primer 5'-
183 GAGGACTCGAGCTCAAGC -3', and ExTaq DNA polymerase. The reaction
184 conditions were 94˚C for 2 min followed by 40 cycles of 94˚C for 0.5 min, 55˚C for 0.5
185 min and 72˚C for 0.5 min with final extension at 72˚C for 5min. For cloning of 5'
186 region pheasant motilin, PCR amplification was performed with 500 ng total RNA, a
187 sense primer 5'-CCGGGTGTGACAAGGAACAAG -3', antisense 5'-
188 GCACTGCCATCACGTACACC-3', and ExTaq DNA polymerase. The reaction
189 conditions were 94˚C for 2 min followed by 40 cycles of 94˚C for 0.5 min, 50˚C for 0.5
190 min and 72˚C for 0.5 min with final extension at 72˚C for 5min.
191 Amplification reactions were carried out using a Thermal Cycler (Bio-Rad, Hercules,
192 California, USA). Amplicon size and specificity were confirmed by 2% agarose gel

193 electrophoresis. The PCR product was cloned into pGEM-T Easy vector (Promega,
194 Madison, WI) and sequencing was performed by Eurofins Genomics K.K (Tokyo,
195 Japan).
196
197 **2.3. Cloning of pheasant ghrelin**
198 Pheasant ghrelin cDNA was determined by 3'- and 5'-RACE PCRs. For 3'-RACE
199 PCR, total RNA (1 µg) from the proventriculus was transcribed with the GeneRacer 3'
200 Oligo-dT Primer using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche
201 Diagnostics GmbH, Mannheim, Germany) (final volume of 20 µl). Primary 3'-RACE
202 PCR was performed with 2 µl of a template, 100 pmol/µl of degenerated primers for a
203 common sequence of ghrelin (GSSFLSP-dg-s1, s2, s3 and s4), 3'-primer and ExTaq
204 DNA polymerase (TaKaRa Bio, Shiga, Japan). The reaction conditions were 94˚C for 2
205 min followed by 35 cycles of 94˚C for 0.5 min, 53˚C for 0.5 min and 72˚C for 1 min
206 with final extension at 72˚C for 3 min. The amplified product was purified by the
207 Wizard PCR Preps DNA Purification System (Promega, Madison, WI), and the
208 resultant product was subjected to second-round nested PCR. Nested PCR was
209 conducted with another 100 pmol/µl of a degenerated sense primer designed by a
210 common sequence of avian ghrelin (**KijiGRL**-dg-s1, 5'-GAA TWT AAA AAM ATA
211 CAG CAA CAA-3') combined with degenerated anti-sense primers (**KijiGRL**-dg-AS1
212 [5'-AGT TTC TTT AGC ATT KTC TTY-3'] and dg-AS2 [5'-KTC TTY RAG AAT
213 GTC CTG TAG-3']) or a 3'-nested primer, PCR-prepsed template and ExTaq DNA
214 polymerase under similar conditions with the primary PCR only modified annealing
215 temperature to 57˚C. The obtained product was subcloned into the pCRII-TOPO vector
216 (Life Technologies Japan), and the nucleotide sequence was determined according to

the protocol of the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems).

To determine the 5'-side cDNA sequence, first-strand cDNAs were synthesized from each 2 µg of proventriculus total RNA with a gene-specific antisense primer (**KijiGRL**-dg-AS1) or oligo dT_{12-18} primer. Primary PCR was conducted using 10 pmol/ µl KijiGRL-AS3 (5'-CTC TTC AAG AAT GTC CTG TAG CAT-3'), a 5'-primer supplied in kit and ExTaq DNA polymerase with amplification conditions of 94˚C for 2 min, followed by 35 cycles of 94˚C for 0.5 min, 56˚C for 0.5 min and 72˚C for 1 min with final extension at 72˚C for 3 min. After purification of the amplified product by PCR preps, second-round nested PCR was performed using **KijiGRL**-AS4 (5'-CTT CTC CAA CGC TTG TCC ATA TTC-3'), a 5'-nested primer and ExTaq DNA polymerase under the same conditions. The obtained nucleotide sequences by the 3'- and 5'-RACE PCRs were assembled and full-length cDNA was finally determined.

2.4. Immunohistochemistry for ghrelin

The pheasants were euthanized by exsanguination via the abdominal aorta under deep anesthesia with 2% isoflurane (Pfizer Japan, Tokyo, Japan). The digestive canal including the esophagus, crop, proventiculus, duodenum, jejunum, ileum, cecum and colon were quickly collected and fixed in Bouin–Hollande fixation solution for 24 h. The fixed tissues were embedded in paraffin, cut into 3-µm-thick sections on a microtome, and mounted on gelatin-coated (super-frost) glass slides. For immunohistochemistry of ghrelin-immuno**reactive** cells, **t**he sections were de-paraffinized with xylene and dehydrated with ethanol. After immersion in deionized water, proteinase K (20 µg/ml, Dako Proteinase K ready-to-use, Dako Cytometion, Kyoto) was dropped on the sections and allowed to incubate for 10 min. After washing

with deionized water **followed by phosphate-buffered saline** (PBS) **(pH 7.4)** (Dainippon-Parma Co. Ltd., Osaka, Japan), the sections were immersed in 1.5% H_2O_2 in methanol for 10 min. After washing with PBS, a blocking solution (Dako Protein Block serum free) was dropped on the sections and allowed to incubate for 30 min. After wiping, anti-octanoylated rat ghrelin **rabbit** serum (1:4000), anti-unacylated ghrelin **rabbit** serum (1:3000) **or** anti-decanoylated rat ghrelin **rabbit** serum (1:2000) (Hiejima et al., 2009) with a diluent (Dako Antibody Diluent with Background Reducing components) were dropped on the sections and allowed to incubate for 16 h at 4˚C in a humid chamber. After washing with PBS, a second antibody solution (Dako Labelled Polymer, HRP Anti-rabbit Envision) was dropped on the sections and allowed to incubate for 30 min at room temperature. After washing with PBS, **the** sections were reacted with 3,3-diaminobenzidine-tetrachloride mixed with 0.012% H_2O_2 in 50 mM Tris-HCl (pH 7.6) for 4 min. After washing with deionized water, counter-staining was carried out with Mayer's hematoxylin. After washing with deionized water, the sections were dehydrated **routinely** and mounted **with malinol (Muto Pure Chemicals Co. Ltd., Tokyo, Japan)**. The sections were viewed under a light microscope (FSX100, OLYMPUS, Tokyo).

2.5. Contraction study for the GI tract of the pheasant

Smooth muscle preparations of different parts of the GI tract from the pheasant were suspended vertically in an organ bath (5 mL) to measure contraction of muscle strips. The organ bath contained warmed (37℃) Krebs solution **equilibrated with 95% O_2 + 5% CO_2 (pH 7.4). Contractile** activity of each isolated muscle preparation was measured with an isometric force transducer, recorded on a computer, and analyzed

using a computer-aided system (Power Lab 2/25, Japan Bioresearch Center, Nagoya, Japan). The 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 motilin and ghrelin, each strip was subjected to 3 or 4 stimulations with 100 μM acetylcholine (ACh) **for 2 min at 15 min interval** until a reproducible contraction was obtained. Increase in smooth muscle tonus by contractile substances among preparations was normalized by a standard contraction of 100 μM ACh and expressed as a relative contraction (%).

To examine the concentration-response relationships of motilin agonists and ACh in respective preparations, erythromycin (1 nM – 10 μM), human motilin (0.1 nM – 3 μM), chicken motilin (0.1 nM – 1 μM) and ACh (1 nM –100 μM) were applied cumulatively in the organ bath after observing the peak response of each concentration (about 2 min interval). The interval for **constructing** the concentration-response relationships **of motilin agonists and ACh** was set at 1 h **to avoid the desensitization of motilin-induced responses**, and the application order of erythromycin, human motilin, chicken motilin or ACh was changed **at random** for each preparation. **Concentration-response curves for motilin were also constructed in the presence of motilin receptor antagonists (GM109 and MA2029), tetrodotoxin (TTX, a neuron blocker) or atropine (a cholinergic muscarinic receptor antagonist) to determine the mechanisms of the motilin-induced contractions.**

To examine the GI contractility stimulating actions of ghrelin, 1 μM rat ghrelin, chicken ghrelin or quail ghrelin (the maximum concentration to check the responsiveness of ghrelin) was applied to the organ bath. Next, to determine the interaction of ghrelin and motilin in the GI tract, chicken motilin was applied

289 cumulatively in the presence of chicken ghrelin (1 μM). In some experiments, the
290 effects of pretreatment with chicken motilin (at concentrations that do not cause
291 contraction [0.3 nM, 3 nM and 10 nM]) on the ghrelin-induced responses in the crop,
292 proventriculus and ileum were also investigated.
293
294 **2.6. Chemicals**
295 The following chemicals were used in the experiments: acetylcholine chloride (Wako,
296 Osaka, Japan), atropine sulphate (Sigma-Aldrich, MO, USA) and tetrodotoxin (Wako).
297 Chicken ghrelin was custom-synthesized by Daiichi Asubio Pharma. Co., Ltd. (Gunma,
298 Japan). Chicken motilin was custom-synthesized by Peptide Institute Inc. (Osaka,
299 Japan). Quail ghrelin was custom-synthesized by Greiner Bio-One Co., Ltd. (Tokyo,
300 Japan). The purity was confirmed by a single peak of reverse-phase HPLC. Human
301 motilin and rat ghrelin were purchased from Peptide Institute Inc. (Osaka, Japan).
302 Erythromycin lactobionate was obtained from U.S. Pharmacopeial Co. Inc. (Rockville,
303 MD, USA). GM109 and MA2029 were kindly donated by Chugai Co. Ltd. (Tokyo,
304 Japan).
305 All chemicals except for MA2029 were dissolved in distilled water and directly
306 applied to an organ bath using a micropipette. The applied volume was less than 0.5%
307 of the bath volume (5 mL). MA2029 was dissolved with dimethysulfoxide (DMSO) and
308 diluted with distilled water at a designated concentration. The maximum concentration
309 of DMSO in the bath was below 0.02%, and this concentration did not affect smooth
310 muscle tonus or motilin-induced contraction.
311
312 **2.7. Statistical analysis**

The experimental data are expressed as means ± SEM of more than four experiments. The significance of differences between the values was determined at $P < 0.05$ using Student's ***t***-test (paired and unpaired) for single comparisons or ANOVA followed by **Dunnett's** test for multiple comparisons by GraphPad Prism6 (GraphPad Software Inc., CA, USA). **Sigmoid curve fitting procedure (GraphPad Prism6) was used calculating the EC_{50} (concentration causing 50% of the maximum contraction) in the present experiments.**

3. Results

3.1. Cloning of pheasant motilin

Pheasant motilin cDNA was cloned from mRNA of the duodenum and its nucleotide sequence was determined (Fig. 1A) (Acc# LC469791.1). The deduced amino acid sequence of pheasant mature motilin was 22 amino acids. Similar to motilin precursors in mammals, an endoproteinase cleavage site was found in pheasant motilin at Lys^{23}-Lys^{24} (Fig. 1A). Mature pheasant motilin showed high sequence homology with other avian species: turkey (100%), chicken (95.4%) and quail (90.9%). **The sequence of N-terminal [1-9] (FVPFFTQSD) , middle region [11-18] (QKMQEKER) and C-terminal of pheasant motilin [(20-22] (KGQ) was the same as that in other birds such as the turkey, chicken and quail (Fig. 1B).** Pheasant motilin showed moderate homology with mammalian species (68% for human and canine motilin and 64% for *Suncus* motilin) (Fig. 1B).

3.2. Cloning of pheasant ghrelin

Pheasant ghrelin cDNA was cloned from mRNA of the proventriculus, and its

nucleotide sequence was determined (Fig. 2A) (Acc# LC459605). The deduced amino acid sequence of pheasant mature ghrelin was 26 amino acids (GSSFLSPAYKNIQQQKDTRKPTGRLH). Pheasant ghrelin showed differences in two amino acids (8 and 23) from those of chicken ghrelin and in three amino acids (17, 22 and 23) from those of Japanese quail ghrelin. Turkey ghrelin is a 28-amino-acid peptide, and within the N-terminal region [1-26], only one amino acid at position 23 was different from that of pheasant ghrelin (Fig. 2B).

3.3. Ghrelin immunohistochemistry in the pheasant GI tract

We used three antibodies for detection of ghrelin **immunoreactive** cells. The antibody for octanoyl ghrelin failed to stain any cells. We then used antibodies for decanoyl ghrelin **or** unacylated ghrelin, and both antibodies were able to stain **scattering** ghrelin-**containing** cells in the mucosa of the proventriculus. The number of decanoyl ghrelin **immunoreactive** cells was comparable to the number of ghrelin **immunoreactive** cells detected by the unacylated ghrelin **antibody (5-6 cells/160 mm^2)** (Fig. 3). **A few ghrelin immunoreactive** cells were detected in the mucosa of the duodenum by the unacylated ghrelin **antibody but not other antibodies** (Fig. 3).

3.4. Effects of chicken motilin on the pheasant GI tract

We examined the contractile activity of chicken motilin instead of pheasant motilin on the pheasant GI tract since the structure of pheasant motilin was close to that of chicken motilin. As shown in Fig. 4, chicken motilin caused a **marked** concentration-dependent contraction in small intestinal preparations (duodenum, jejunum and ileum). Contraction was evoked at 1 - 3 nM and reached a maximum at 100 – 300 nM. The

361 EC_{50} values and the maximum amplitude (% to 100 μM ACh-induced contraction) were
362 20.1 ± 6.8 nM and 79.1 ± 7.9% in the duodenum (n = 8), 30.6 ± 10.8 nM and 73.9 ±
363 6.0% in the jejunum (n = 5), and 26.4 ± 7.6 nM and 88.30 ± 10.3% in the ileum (n =
364 17), respectively. On the other hand, other GI regions such the crop, proventriculus and
365 colon were less sensitive to chicken motilin. The contractile responses in the
366 proventriculus reached a significant level at 300 nM and 1 μM compared with the
367 normal muscle tonus (Fig. 4), but the muscle tonus in the crop and colon did not reach a
368 significant level even at 1 μM **compared with that in the absence of chicken motilin**
369 **(Dunnett's test)**. ACh (1 nM – 100 μM) caused a concentration-dependent contraction
370 of all parts of the pheasant GI tract, and the EC_{50} values were comparable among the GI
371 regions examined (EC_{50} values: 760.6 ± 487.3 nM for the crop (n = 4), 533.8 ± 158.0
372 nM for the proventriculus (n = 7), 396.8 ± 134.2 nM for the duodenum (n = 5), 225.8 ±
373 62.4 nM for the jejunum (n = 5), 442.2 ± 153.4 nM for the ileum (n = 6) and 343.3 ±
374 114.8 nM for the colon (n = 6)).

375 Human motilin also **caused contraction** in the small intestinal preparations of the
376 pheasant (Fig. 5). The maximum responses to human motilin were comparable to those
377 to chicken motilin in the three intestinal regions, but the EC_{50} values (276.4 ± 22.7 nM
378 for the duodenum (n = 4), 227.5 ± 63.1 nM for the jejunum (n = 6) and 117.3 ± 32.8 nM
379 for the ileum (n =7)) were **significantly** higher than those of chicken motilin **(Student's**
380 **unpaired *t*-test)**. Human motilin-induced responses were smaller than those of chicken
381 motilin in the proventriculus, and they were not significant even at 1 μM (**Dunnett's**
382 **test)**. As was observed for chicken motilin, human motilin did not cause contraction in
383 the crop and colon. Erythromycin, a motilin receptor agonist in mammalian GI tracts
384 (Peeters et al., 1989) (1 nM – 10 μM) did not cause any **contractions** in the pheasant

385 proventriculus, duodenum, jejunum and ileum (Fig. 5).

386

387 **3.5. Mechanisms of motilin-induced GI contraction**

388 **To investigate the involvement of mammalian-like motilin receptor in the**
389 **motilin-induced contraction of the pheasant GI tract, we investigated the effects of**
390 **two mammalian motilin receptor antagonists with different affinity, GM109 and**
391 **MA2029 (Takanashi et al., 1995; Sudo et al., 2008).** Pretreatment of GM109 (1 μM)
392 alone did not cause any **contractions** and did not **change** the contractile responses to
393 motilin in the proventriculus and ileum (Figs. 6A and 6B). The EC_{50} value (35.6 ± 11.9
394 nM, n = 11) and the maximum contraction (92.3 ± 9.7%, n = 11) of the ileum in the
395 presence of GM109 were comparable to those in the control **(Student's unpaired *t*-**
396 **test)**. Pretreatment with MA2029 (1 μM) **also did not inhibit** the contractile responses
397 to chicken motilin in the ileum (EC_{50} = 14.3 ± 8.3 nM, maximum response = 92.6 ±
398 5.5%, n = 4) (Fig. 6B) **(Student's unpaired *t*-test)**.

399 To examine the mechanisms underlying the motilin-induced contraction, the effects of
400 tetrodotoxin (TTX) and atropine on the responses to chicken motilin were investigated.
401 As shown in Fig. 7B, motilin-induced contraction in the ileum was not affected by
402 pretreatment with TTX (1 μM). The EC_{50} values (33.0 ± 12.8 nM, n=10) and maximum
403 contractile amplitudes (73.1 ± 6.9%, n = 10) were **almost same** with those of the
404 control **(Student's unpaired *t*-test)**. The contractile responses to chicken motilin in the
405 duodenum and jejunum were also not decreased by treatment with TTX (EC_{50} and
406 maximum contraction, duodenum; 53.1±15.7 nM and 69.60 ± 14.6%, n = 9, jejunum;
407 46.1 ± 20.4 nM and 58.3 ± 10.4%, n = 10). On the other hand, the motilin-induced
408 responses in the proventriculus were decreased by TTX (Fig. 7A). The relative

amplitude of contraction at 1 μM of chicken motilin (3.3 ± 1.0%) (n = 3) was significantly smaller than that of the control value (13.6 ± 2.9%, n = 14) **(Student's unpaired *t*-test)**. The effects of atropine on the motilin-induced contraction were the same as those of TTX: atropine (1 μM) did not affect chicken motilin-induced contraction of the ileum (EC_{50} and maximum contraction: 14.2 ± 4.3 nM and 75 ± 4.0%, respectively) (n = 11), whereas it significantly decreased the contraction induced by chicken motilin in the proventriculus (5.4 ± 1.4%, n = 7) **(Student's unpaired *t*-test)** (Fig. 7).

3.6. Effects of ghrelin on GI motility

Figure 8 shows typical mechanical responses to ghrelin in the pheasant **GI tracts.** Rat, chicken and quail ghrelins (1 μM), did not cause any **contractility** changes in the crop, proventriculus, ileum **and colon (Fig. 8)**. The relative increases in muscle tonus caused by rat, chicken and quail ghrelins were 0.0 ± 0.2%, 0.1 ± 0.1% and 2.5 ± 1.6% in the crop (n = 6), and 1.3 ± 0.7%, 2.5 ± 1.8% and 1.7 ± 1.1% in the proventriculus (n = 6), 1.5 ± 1.0%, 5.6 ± 2.7% and 3.1 ± 4.0% in the ileum (n = 6) **and 2.2 ± 1.4%, 2.5 ± 0.3%, 1.9 ± 1.3% in the colon (n = 4), respectively. There were not significant differences in the muscle contractility between absence and presence of ghrelins (Student's paired *t*-test).**

3.7. Possible interaction of ghrelin and motilin in the contractile response

The effects of pretreatment with chicken ghrelin on the responses to chicken motilin in the proventriculus and ileum were examined. Concentration-response curves of chicken motilin did not change in the presence of chicken ghrelin (1 μM) in both GI

433 preparations. In addition, the crop was also insensitive to chicken motilin even in the
434 presence of 1 μM chicken ghrelin (n = 4) (Fig. 9) **(Student's unpaired *t*-test)**. Quail
435 ghrelin (1 μM) treatment also did not change the responses to chicken motilin in the
436 proventriculus and ileum (data not shown).

437 We also examined the effects of pretreatment with low concentrations of chicken
438 motilin (0.3, 3 and 10 nM in the crop and proventriculus) on the ghrelin-induced
439 responses. These concentrations of chicken motilin did not cause any **contractility**
440 changes in the respective preparations. Pretreatment with chicken motilin (0.3, 3 and 10
441 nM) did not affect the **contractility** to successively applied chicken ghrelin (1 μM) in
442 the crop and proventriculus (Fig. 10). Chicken ghrelin (1 μM) also caused no
443 contraction of the ileum in the presence of 0.3 nM chicken motilin. In the chicken
444 motilin pretreated crop, proventrsiculus and ileum, quail ghrelin (1 μM) was also
445 ineffective causing the contraction (data not shown).

446

447 **4. Discussion**

448 This study showed that both the motilin and ghrelin systems are present in the
449 pheasant as was found in chicken and quail. The GI region-dependent contractions
450 induced by motilin with different mechanisms in the proventriculus and ileum were the
451 same as those reported for the chicken (Kitazawa et al., 1997) and quail (Apu et al.,
452 2016). On the other hand, ghrelin did not induce contraction in any GI regions.
453 Interaction of motilin and ghrelin observed in the *Suncus*（Mondal et al., 2012）was
454 not observed in the pheasant ***in vitro***. Therefore, it is likely that involvement of the
455 motilin system, but not the ghrelin system, **in regulation of the GI contractility** is the
456 common feature in avian species.

457

458 **4.1. Pheasant motilin and its action on GI contractility**

459 **We first determined the deduced mature sequence of pheasant motilin, FVPFF**
460 **TQSDI QKMQE KERIK GQ, and its structure was the same as that of turkey**
461 **motilin, and only one amino acid was different from that of chicken or quail**
462 **motilin.** In mammals, the N-terminal amino acid sequence of motilin is quite important
463 and it was found to be capable of affecting full agonistic activity when examined using
464 cell lines that overexpressed the motilin receptor, GPR38 (Poitras et al., 1992). The first
465 eight amino acids (FVPFFTQS), which comprise an important N-terminal structure for
466 the activity of motilin, were identical among avian species (pheasant, chicken, turkey
467 and quail). In the case of mammalian motilin, human motilin was the same as porcine
468 motilin, but canine motilin was different in five amino acids and *Suncus* motilin and
469 rabbit motilin were different in **three or four** amino acids **in 22 amino acids**,
470 respectively, from those of human motilin **(Itoh, 1997; Tsutusi et al., 2009; Kitazawa**
471 **and Kaiya, 2019). Compared with a marked species-related variation of motilin**
472 **structure in mammals, the species difference is quite small in avian species**
473 **examined so far.**

474 Since the structure of chicken motilin is close to that of pheasant motilin, chicken
475 motilin was used for the contraction study. As expected, chicken motilin contracted the
476 pheasant GI tract. The small intestine was much more sensitive to chicken motilin than
477 was the proventriculus, crop and colon. This is consistent with the results for chickens
478 and quails (Kitazawa et al., 1997; 2007; Apu et al., 2016). Therefore, region-dependent
479 different responsiveness to motilin is a common feature in avian GI tract. The GI
480 regional difference in motilin response is due to heterogeneous expression of the motilin

481 receptor (Kitazawa et al., 2013). The high sensitivity of the small intestine to motilin
482 suggests that the small intestine **might be** the main target of motilin in birds. In fact,
483 motilin has been shown to be a mediator of rhythmic oscillatory contraction in the
484 chicken small intestine (Rodríguez-Sinovas et al., 1997).

485 GM109 and MA2029 are known to be mammalian motilin receptor antagonists, and
486 their **pK_d values (binding affinity)** for the rabbit duodenal motilin receptor were 7.34
487 for GM109 and 9.17 for MA2029 (Takanashi et al., 1995; Sudo et al., 2008). GM109
488 and MA2029 have been shown to decrease motilin-induced responses in the rabbit
489 duodenum (Takanashi et al., 1995; Sudo et al., 2008; Kitazawa et al., 2017b). However,
490 in this study, GM109 and MA2029 did not decrease the responses to chicken motilin in
491 the pheasant proventriculus or ileum. The insensitivity of GM109 **to decrease the**
492 **motilin response** in the pheasant is the same as that reported in the chicken GI tract
493 (Kitazawa et al., 1997). Homology of the chicken motilin receptor with human and
494 rabbit motilin receptors has been reported to be 59% and 65%, respectively (Yamamoto
495 et al., 2008). On the other hand, the homologies of mammalian motilin receptors to the
496 human motilin receptor are considerably high (rabbit: 84%, *Suncus*: 76%, dog: 71%)
497 (Dass et al., 2003a; Ohshiro et al., 2008; Suzuki et al., 2012). Human motilin also
498 caused contraction of the small intestine of the pheasant, but its sensitivity was lower
499 than that of chicken motilin as reported in the chicken GI tract (Kitazawa et al., 1997)
500 and it was also lower than the sensitivity in the rabbit duodenum (Kitazawa et al.,
501 1994). In addition, erythromycin, a motilin receptor agonist in mammals (Peeters et al.,
502 1989), also did not cause contraction even at 10 μM. These results suggest that the
503 different structure of the pheasant motilin receptor from that of the human motilin
504 receptor affects the affinity of the motilin receptor agonists and antagonists, although

505 the **structure** of the pheasant motilin receptor has not yet been determined.

506 The mechanisms of motilin-induced contraction were characterized using atropine
507 and TTX. Motilin-induced contractions in the small intestine were not attenuated by
508 atropine or TTX, but those in the proventriculus were decreased by each blocker. **TTX,**
509 **a Na^+ channel blocker, decreases the neural responses in smooth muscle**
510 **preparations and atropine is an antagonist of muscarinic cholinergic receptors.**
511 The present results suggest **that motilin acts the motilin receptors on the smooth**
512 **muscle cells of the small intestine, whereas it acts** on the neural receptors **located on**
513 **cholinergic enteric neurons** in the proventriculus, as demonstrated in chickens and
514 quails (Kitazawa et al., 1997; Apu et al., 2016). Therefore, **it was suggested that**
515 mechanisms of motilin-induced contraction are different in the proventriculus and small
516 intestine, and the region-dependent different contractile mechanisms of motilin are a
517 common characteristic of avian GI tracts.

518

519 **4.2. Pheasant ghrelin and its action on GI contractility**

520 Pheasant ghrelin cloned in the present study was a 26-amino-acid peptide, GSSFL
521 SPAYK NIQQQ KDTRK PTGRLH. Compared with the structures in other birds, the
522 N-terminal [1-7] (GSSFLSP) sequence is completely conserved in all birds, but the
523 overall pheasant ghrelin sequence is different from chicken ghrelin at positions 8 and 23
524 and is different from quail ghrelin at positions 17, 22 and 23. The structure of turkey
525 motilin is the same as that of pheasant motilin in this study. When pheasant ghrelin was
526 compared with turkey ghrelin, only one amino acid at position 23 was different within
527 the 26 amino acids sequences, though turkey ghrelin is composed of 28 amino acids in
528 total. The structural similarity is due to the close phylogenetic position between

529 pheasants and turkeys.

530 In an immunohistochemical study, ghrelin-**immunoreactive** cells were detected in
531 the mucosal layer of the proventriculus, and their shape was a round, closed-type as
532 observed in chickens (Wada et al., 2003; Yamato et al., 2005). Interestingly, the ghrelin-
533 **immunoreactive** cells were stained by a specific antibody **for** decanoyl ghrelin but not
534 **for** octanoyl ghrelin, suggesting that Ser-3 of the pheasant ghrelin is likely to be
535 acylated by decanoic acid. In the case of chickens, Ser-3 of ghrelin was modified by
536 both octanoic acid and decanoic acid (Kaiya et al., 2002). **Ghrelin-immunoreactive**
537 cells were also detected in the duodenum by an antibody for unacylated ghrelin but not
538 by antibodies for octanoyl and decanoyl ghrelin, suggesting that duodenal ghrelin is not
539 acylated. In addition, the cell shape was an **elongated**-type, which was observed in
540 intestinal **ghrelin-immunoreactive** cells in the chicken and rainbow trout (Wada et al.,
541 2003; Sakata et al., 2004).

542 In the pheasant GI tract, three ghrelins (rat, chicken and quail ghrelins) at 1 μM did
543 not cause any contraction of the crop, proventriculus, ileum **and colon**. The actions of
544 ghrelin in the avian GI tract were contrastive between the chicken and quail (Kitazawa
545 et al., 2007, 2009; Apu et al., 2016). Chicken ghrelin caused contraction of the chicken
546 proventriculus and crop, but there were no responses in the same regions of the quail GI
547 tract despite the expression levels of ghrelin receptor mRNA being almost the same
548 (Kitazawa et al., 2009). Therefore, the response to ghrelin in the pheasant GI tract was
549 similar to that in the quail **not in the chicken.** These results including the results of this
550 study suggest that **regulation** of GI motility by ghrelin varies **even** among **closely**
551 **related** avian species.

552

4.3. Interaction of motilin and ghrelin

An interaction of ghrelin and motilin has been reported in the *Suncus* and dogs. ghrelin caused contraction of the *Suncus* stomach in the presence of a low concentration of motilin, while ghrelin was ineffective in the absence of motilin (Mondal et al., 2012). In conscious dogs, ghrelin inhibited the motilin-mediated **phase-III of MMC** despite the fact that ghrelin alone did not induce any **contractions in phase-I of MMC** (Ogawa et al., 2012). The presence of ghrelin and motilin systems has been demonstrated in chickens and quails, but interaction of ghrelin and motilin was **only examined** in the quail intestine (Apu et al., 2016). In the present experiments, chicken motilin-induced contraction was not affected by pretreatment with quail ghrelin or chicken ghrelin in the peasant proventriculus and ileum. In addition, a low concentration of chicken motilin that does not cause any contraction did not modify the actions of ghrelin in the crop, proventriculus and ileum. These results suggested that there is no interaction between motilin and ghrelin in the pheasant GI tract as is the case in the quail (Apu et al., 2016) at least in an *in vitro* condition.

4.4. Conclusion

In this study, the presence of both motilin and ghrelin was demonstrated in the pheasant. Motilin caused contraction of the GI tract in a region-dependent manner, but ghrelin was ineffective **causing contractions**. The results indicate that ghrelin-related modulation of GI motility as observed in chickens **might not be** common in avian species. On the other hand, **although physiological experiments are restricted in closely related avian species,** the results suggested that motilin is the common **regulator** of GI **contractility** in birds. **However, further studies using different avian**

species different from chicken, quail and pheasant are needed in future to establish the physiological roles of motilin in avian GI tract.

The authors declare no conflict of interest.

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787 **Figure legends**

788 **Fig. 1. Pheasant motilin structure.** (A): Nucleotide sequence encoding pheasant
789 motilin precursor. The nucleotide sequence has been deposited in the
790 DDBJ/EMBL/GenBank databases with the Accession No. LC469791.1. Mature motilin
791 peptide is boxed, and a dibasic cleavage site (Lys-Lys) is indicated by bold letters and
792 underline. (B): Comparison of amino acid sequences of mature motilin in some birds
793 and mammals. Conserved amino acids among all species are indicated by asterisks
794 (*).The amino acid sequence of pheasant motilin (LC469791.1) was aligned with those
795 of human (AAI12315.1), dog (NP_001300735.1), Suncus (BAI66099.1), turkey
796 (XP_010722636.1), chicken (NP_001292058.1) and quail (BAU80773.1) motilins.
797
798 **Fig. 2. Pheasant ghrelin structure.** (A). Nucleotide sequence encoding pheasant
799 ghrelin precursor. The nucleotide sequence has been deposited in the
800 DDBJ/EMBL/GenBank databases with the Accession No. LC459605. Mature ghrelin
801 peptide is boxed, and a dibasic cleavage site (Arg-Arg) is indicated by bold letters and
802 underline. (B). Comparison of amino acid sequences of mature ghrelin in some birds.
803 Conserved amino acids among all species are indicated by asterisks (*). The amino
804 acid sequence of ghrelin was aligned with those of the chicken (AB075215), duck
805 (AY338466), emu (AY338467), goose (AY338465), Japanese quail (AB244056) and
806 turkey (AY333783).
807
808 **Fig. 3. Ghrelin-immunoreactive cells in the proventriculus and duodenum of the**
809 **pheasant.** A: Proventriculus. Arrows indicate **immunoreactive** cells stained by
810 antiserum that recognizes anti-unacylated ghrelin; B: Proventriculus. Arrows indicate
811 immuno**reactive** cells stained by antiserum that recognizes decanoylated ghrelin; C:

812 Duodenum. Arrows indicate immunoreactive cells stained by antiserum that recognizes
813 anti-unacylated ghrelin; D: Proventriculus stained by normal rabbit serum (negative
814 control).
815
816 **Fig. 4. Contractile responses to chicken motilin in different regions of the pheasant**
817 **GI tract**. (A): Representative mechanical responses to chicken motilin in the crop,
818 proventriculus, duodenum, jejunum, ileum and colon. Chicken motilin was applied
819 cumulatively (0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 nM). Arrowheads indicate the
820 timing of motilin application. (B): Comparison of concentration-response curves by
821 chicken motilin in the crop (●), proventriculus (■), duodenum (▲), jejunum (▼),
822 ileum (◆) and colon (○). The amplitude of motilin-induced contractions (y-axis) was
823 normalized by a standard contraction by ACh (100 μM). The X-axis is the concentration
824 of motilin (logM). Values are means ± S.E.M (n=4-17). **Among less sensitive GI**
825 **regions (crop, proventriculus and colon) to motilin,** the increase of muscle **tonus in**
826 **the proventriculus** was significant compared with that in the absence of chicken
827 motilin (*, $p<0.05$), whereas the responses of **1 μM chicken motilin** in the crop and
828 colon were not significant **compared with those in absence of motilin (Dunnett's**
829 **test).**
830
831 **Fig. 5. Comparison of contractile responses to chicken motilin, human motilin and**
832 **erythromycin in the proventriculus and small intestine.** The symbols indicate the
833 concentration-response curves for chicken motilin (●), human motilin (■) and
834 erythromycin (▲) in the proventriculus (A), duodenum (B), jejunum (C) and ileum (D).
835 The amplitude of contractile responses (y-axis) was normalized by a standard

836 contraction by ACh (100 μM). The x-axes are concentrations of reagents (logM).
837 Values are means ± S.E.M (n=4-17).
838
839 **Fig. 6. Effects of mammalian motilin receptor antagonists on contractile responses**
840 **to chicken motilin in the proventriculus and ileum of the pheasant.** (A):
841 Concentration-response curves of chicken motilin in the absence (control,●) and
842 presence of GM109 (1 μM, ■) in the proventriculus. (B): Concentration-response
843 curves of chicken motilin in the absence (control,●) and presence of GM109 (1 μM,
844 ■) or MA2029 (1 μM, ▲) in the ileum. The amplitude of contractile responses (y-
845 axis) was normalized by a standard contraction by ACh (100 μM). The x-axes are
846 concentrations of reagents (logM). Values are means ± S.E.M (n=4-17).
847
848 **Fig. 7. Effects of atropine and tetrodotoxin on contractile responses to chicken**
849 **motilin in the proventriculus and ileum of the pheasant.** The symbols indicate
850 concentration-response curves for chicken motilin (A: proventriculus, B:ileum) in the
851 absence (control,●) and presence of tetrodotoxin (1 μM, ▲) or atropine (1 μM, ■).
852 The amplitude of contractile responses (y-axis) was normalized by a standard
853 contraction by ACh (100 μM). The x-axes are concentrations of reagents (logM).
854 Values are means ± S.E.M (n=4-17). *, # $P<0.05$; compared with corresponding control
855 responses to chicken motilin.
856
857 **Fig. 8. Representative effects of rat, quail and chicken ghrelins on spontaneous**
858 **contractility of the crop, proventriculus, ileum and colon.** Each ghrelin at 1 μM was
859 applied at the mark (●) and effects were observed **for 5min**.

Fig. 9. Effects of treatment with chicken ghrelin on chicken motilin-induced responses in the crop, proventriculus and ileum. Concentration-response curves for chicken motilin were constructed in the crop (A), proventriculus (B) and ileum (C) in the absence (control, ●) and presence of chicken ghrelin (1 μM, ■). The amplitude of contractile responses (y-axis) was normalized by a standard contraction by ACh (100 μM). The x-axes are concentrations of reagents (logM). Values are means ± S.E.M (n=4-6).

Fig. 10. Representative effects of pretreatment with chicken motilin on the ghrelin-induced mechanical responses in the crop and proventriculus.

The crop and proventriculus were treated with three different concentrations of chicken motilin (0.3 nM, 3 nM and 10 nM) for 5 min and then chicken GHRELIN (1 μM) was added to observe contractile responses. **Pretreatment time (5 min) was enough for appearance of motilin-induced responses.**

Fig. 1

A

```
1   ATGGTTTCGAAGAAGGCGGCGTCCGGTTTGCTCCTGGTGTACGTG
    M  V  S  K  K  A  A  S  G  L  L  L  V  Y  V  15

46  ATGTCAGTGCTGGCAGAACGGGCTGAAGGCTTTGTGCCCTTCTTC
    M  S  V  L  A  E  R  A  E  G  F  V  P  F  F  30

91  ACTCAGAGCGACATCCAGAAAATGCAGGAAAAGGAGAGGATCAAA
    T  Q  S  D  I  Q  K  M  Q  E  K  E  R  I  K  45

136 GGGCAGAAGAAATCCCTGACCTCTCTGCAGCAGCTGGAAGAGGAA
    G  Q  K  K  S  L  T  S  L  Q  Q  L  E  E  E  60

181 GGCTTCTCTGAACAATCTGGTGCAGATAACGAGGGGATGAAGACT
    G  F  S  E  Q  S  G  A  D  N  E  G  M  K  T  75

226 ATCCAGCTAGCTGTCCCTGTCAGGGCTGGGATGTGGCTCATACTG
    I  Q  L  A  V  P  V  R  A  G  M  W  L  I  L  90

271 AGGCAGCTGGAAAAATACCAAGGTGTCCTGGAGAAACTGCTCACG
    R  Q  L  E  K  Y  Q  G  V  L  E  K  L  L  T  105

316 GAGGTGTTACAGGACACCCCAAACGCTGACTGA
    E  V  L  Q  D  T  P  N  A  D  *  115
```

B

```
Pheasant    1   FVPFFTQSDIQKMQEKERIKGQ 22
Turkey      1   FVPFFTQSDIQKMQEKERIKGQ 22
Chicken     1   FVPFFTQSDIQKMQEKERNKGQ 22
Quail       1   FVPFFTQSDFQKMQEKERNKGQ 22
Suncus      1   FMPIFTYGELQKMQEKEQNKGQ 22
Human       1   FVPIFTYGELQRMQEKERNKGQ 22
Dog         1   FVPIFTHSELQKIREKERNKGQ 22
                * * **    *   ***  ***
```

Fig. 2

A

```
  1 ATGTTTCTCAGAGTTGCTCTGCTAGGAATTCTCCTTCTCAGCATCCTCGGGACAGAAACT
    M  F  L  R  V  A  L  L  G  I  L  L  L  S  I  L  G  T  E  T   20

 61 GCTCTGGCTGGCTCCAGTTTTTTAAGCCCCGCATATAAAAACATACAGCAACAAAAGGAT
    A  L  A  G  S  S  F  L  S  P  A  Y  K  N  I  Q  Q  Q  K  D   40

121 ACAAGAAAACCAACAGGAAGATTACATCGCAGAGGCACAGAAAGCTTTTGGGATACAGAT
    T  R  K  P  T  G  R  L  H  R  R  G  T  E  S  F  W  D  T  D   60

181 GAAACAGAAGGAGAAGATGACAATAACAGCCTTGATATCAAGTTTAATGTTCCTTTTGAA
    E  T  E  G  E  D  D  N  N  S  L  D  I  K  F  N  V  P  F  E   80

241 ATTGGTGTCAAGATAACAGAAAGAGAGTATCAAGAATATGGACAAGCGTTGGAGAAGATG
    I  G  V  K  I  T  E  R  E  Y  Q  E  Y  G  Q  A  L  E  K  M  100

301 CTACAGGACATTCTTGAAGAGAATGCTAAAGAAATTCTGACAAAAGACTAA  351
    L  Q  D  I  L  E  E  N  A  K  E  I  L  T  K  D  *
```

B

```
Pheasant          1  GSSFLSPAYKNIQQQKDTRKPTGRLH--   26
Chicken           1  GSSFLSPTYKNIQQQKDTRKPTARLH--   26
Duck              1  GSSFLSPEFKKIQQQNDPTKTTAKIH--   26
Emu               1  GSSFLSPDYKKIQQRKDPRKPTTKLH--   26
Goose             1  GSSFLSPEFKKIQQQNDPAKATAKIH--   26
Japanese quail    1  GSSFLSPAYKNIQQQKNTRKPAARLH--   26
Turkey            1  GSSFLSPAYKNIQQQKDTRKPTARLHPR   28
                     *******  * ***     *     *
```

Fig.3

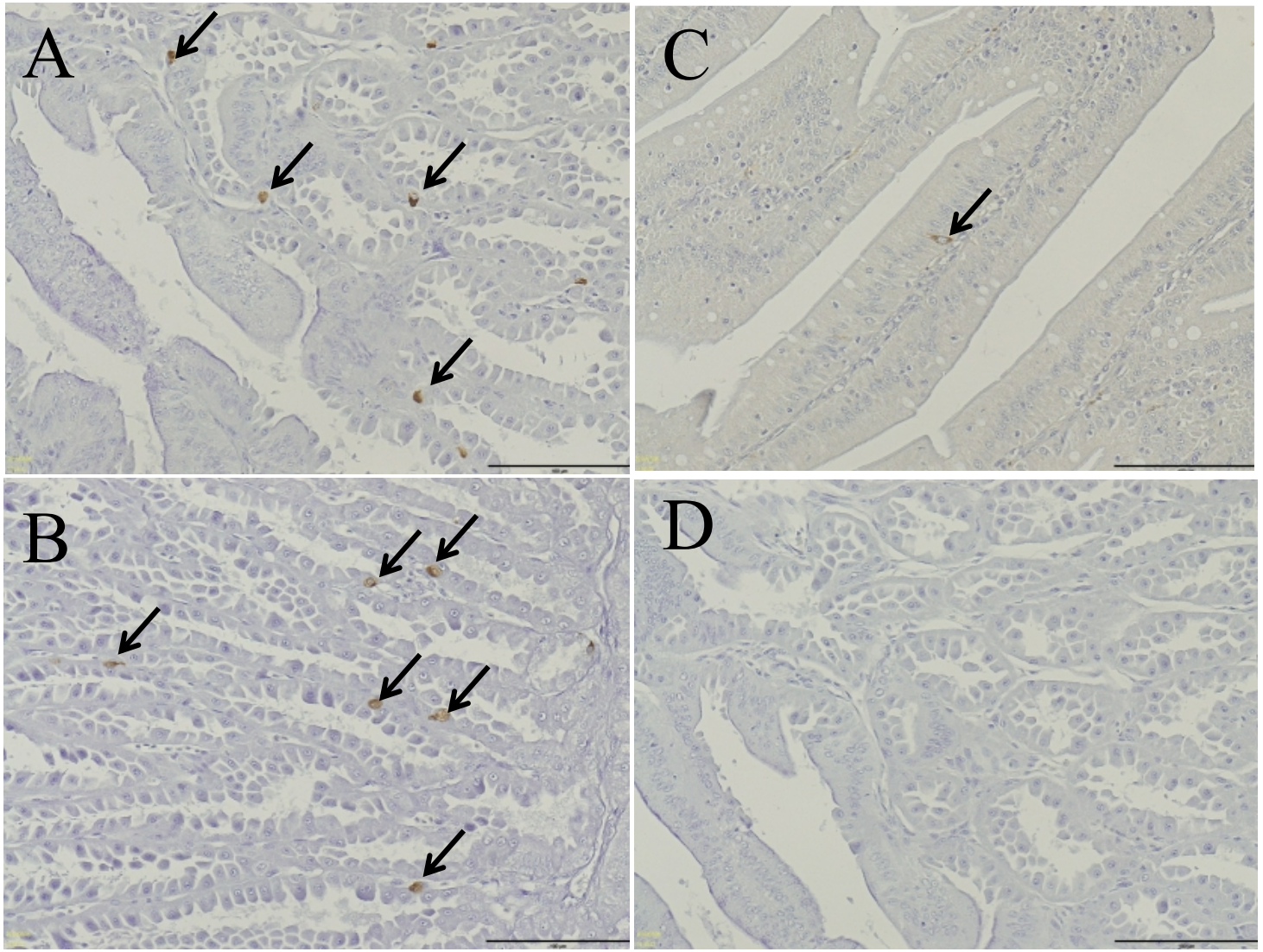
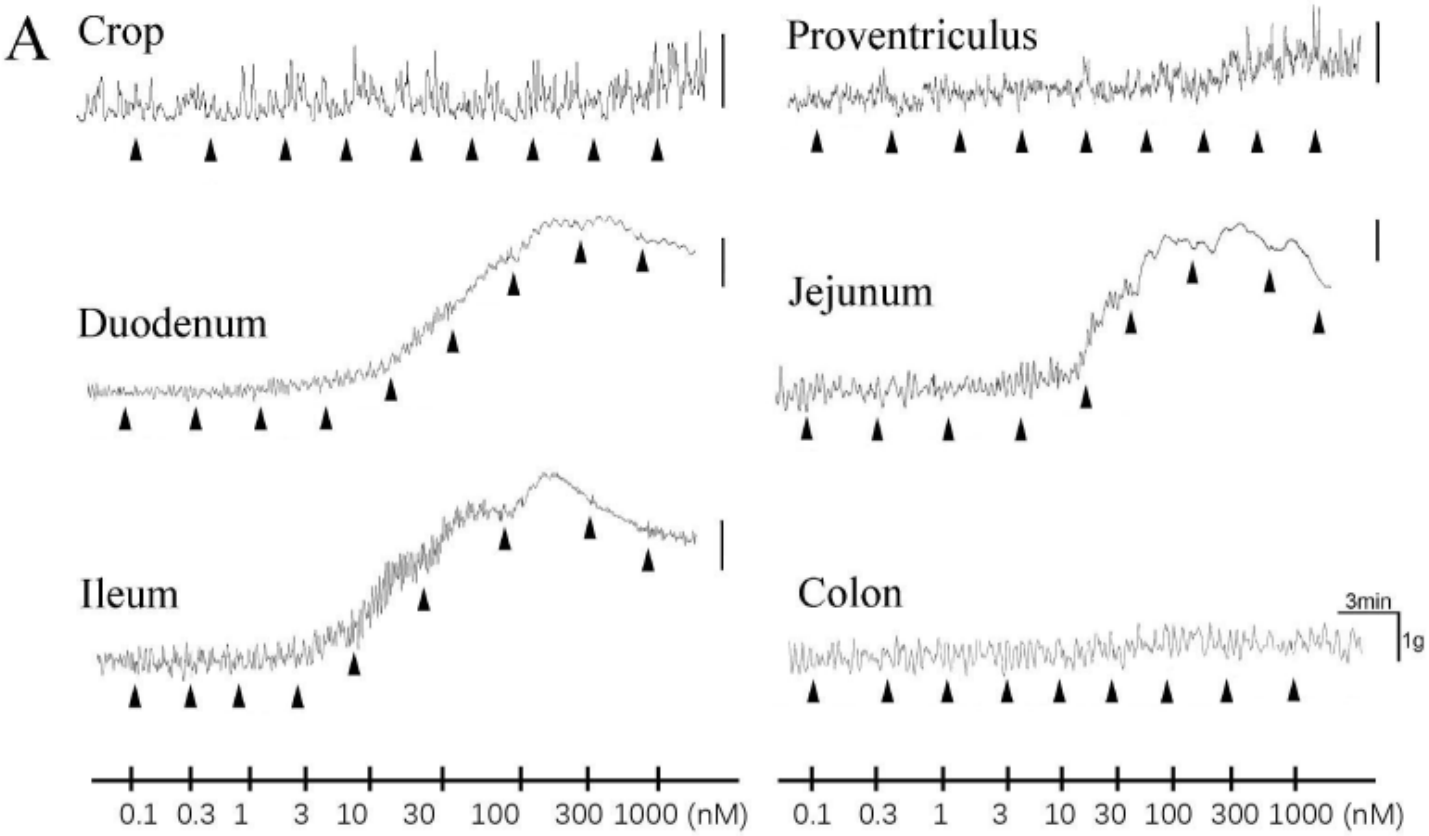


Fig.4


A
Crop
Proventriculus
Duodenum
Jejunum
Ileum
Colon
3min
1g
0.1 0.3 1 3 10 30 100 300 1000 (nM)
0.1 0.3 1 3 10 30 100 300 1000 (nM)

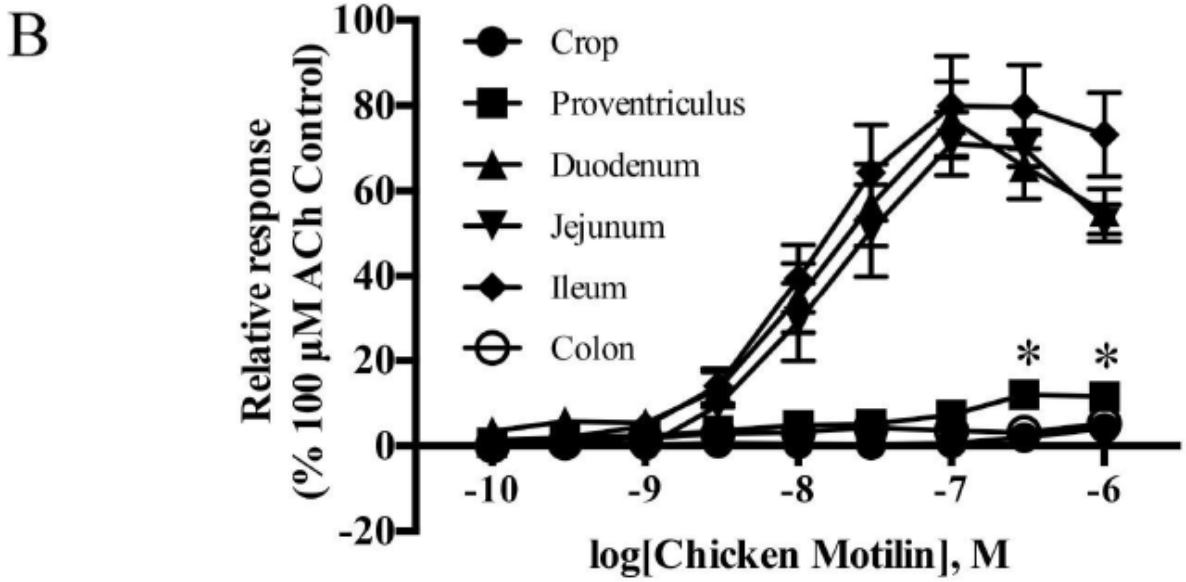

B
Relative response
(% 100 μM ACh Control)
100
80
60
40
20
0
-20
Crop
Proventriculus
Duodenum
Jejunum
Ileum
Colon
*
*
-10
-9
-8
-7
-6
log[Chicken Motilin], M

Fig. 5

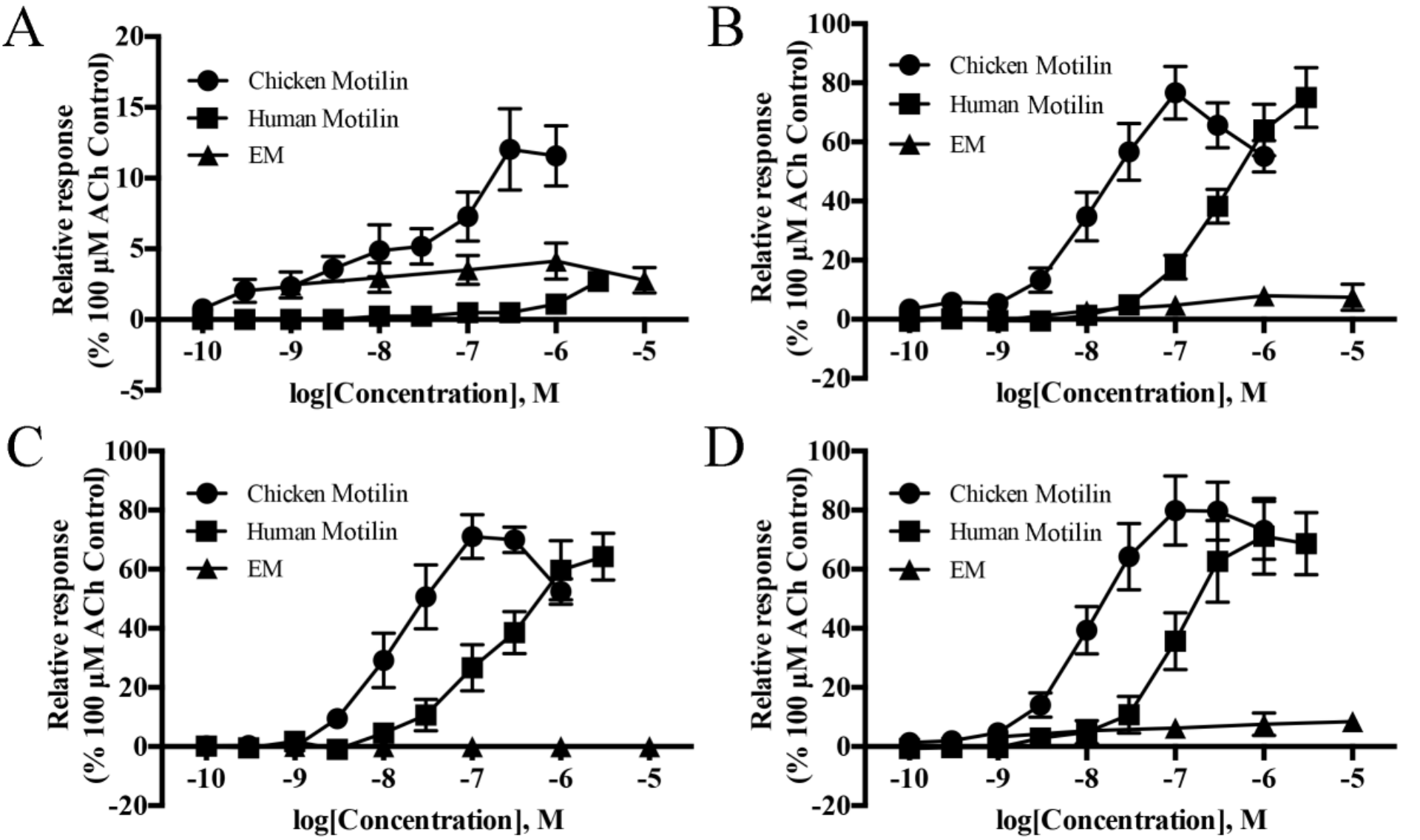

A
B
C
D
Chicken Motilin
Human Motilin
EM
Relative response
(% 100 μM ACh Control)
log[Concentration], M

Fig. 6

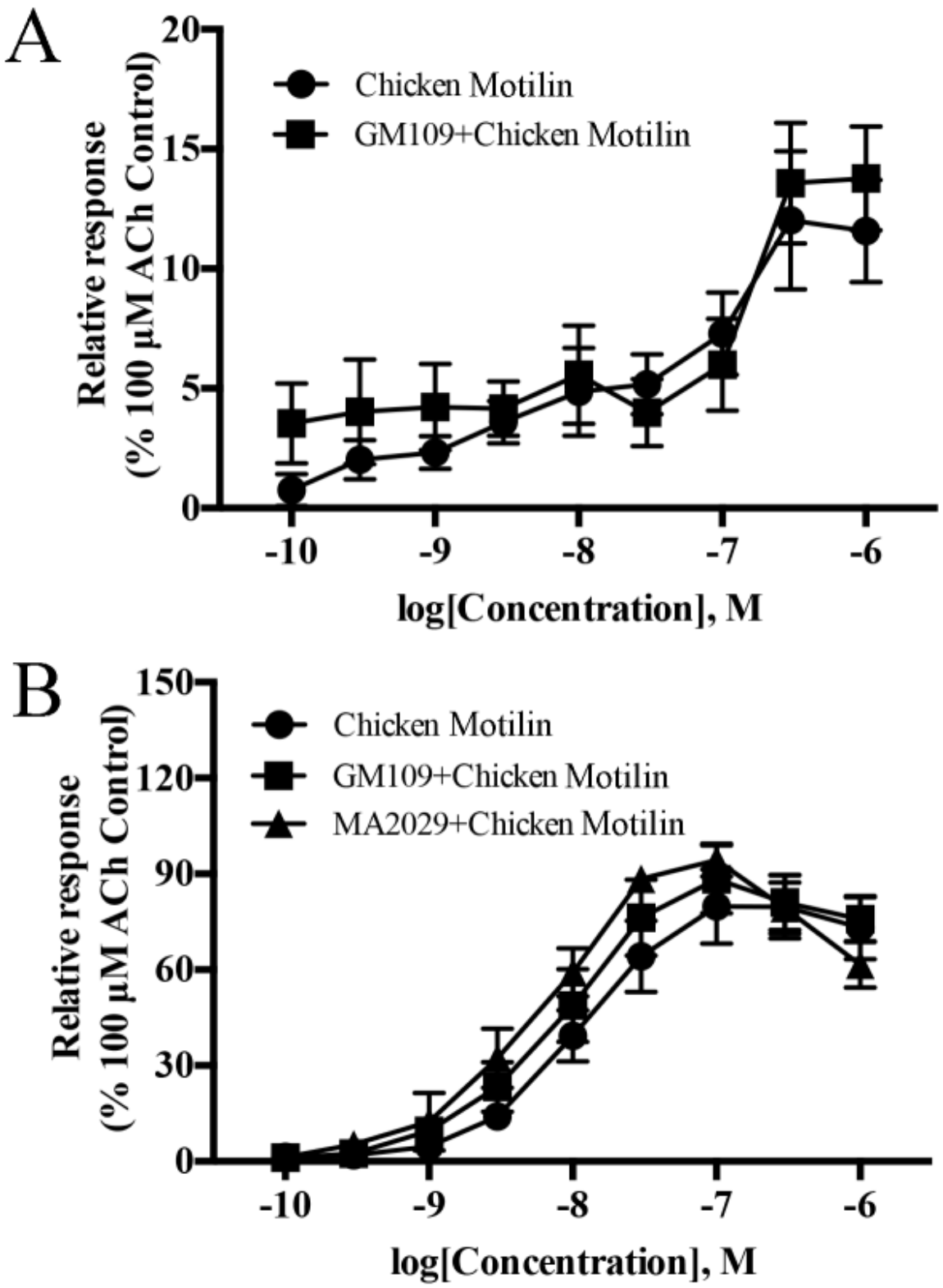

A
Relative response
(% 100 μM ACh Control)
20
15
10
5
0
Chicken Motilin
GM109+Chicken Motilin
-10
-9
-8
-7
-6
log[Concentration], M
B
Relative response
(% 100 μM ACh Control)
150
120
90
60
30
0
Chicken Motilin
GM109+Chicken Motilin
MA2029+Chicken Motilin
-10
-9
-8
-7
-6
log[Concentration], M

Fig. 7

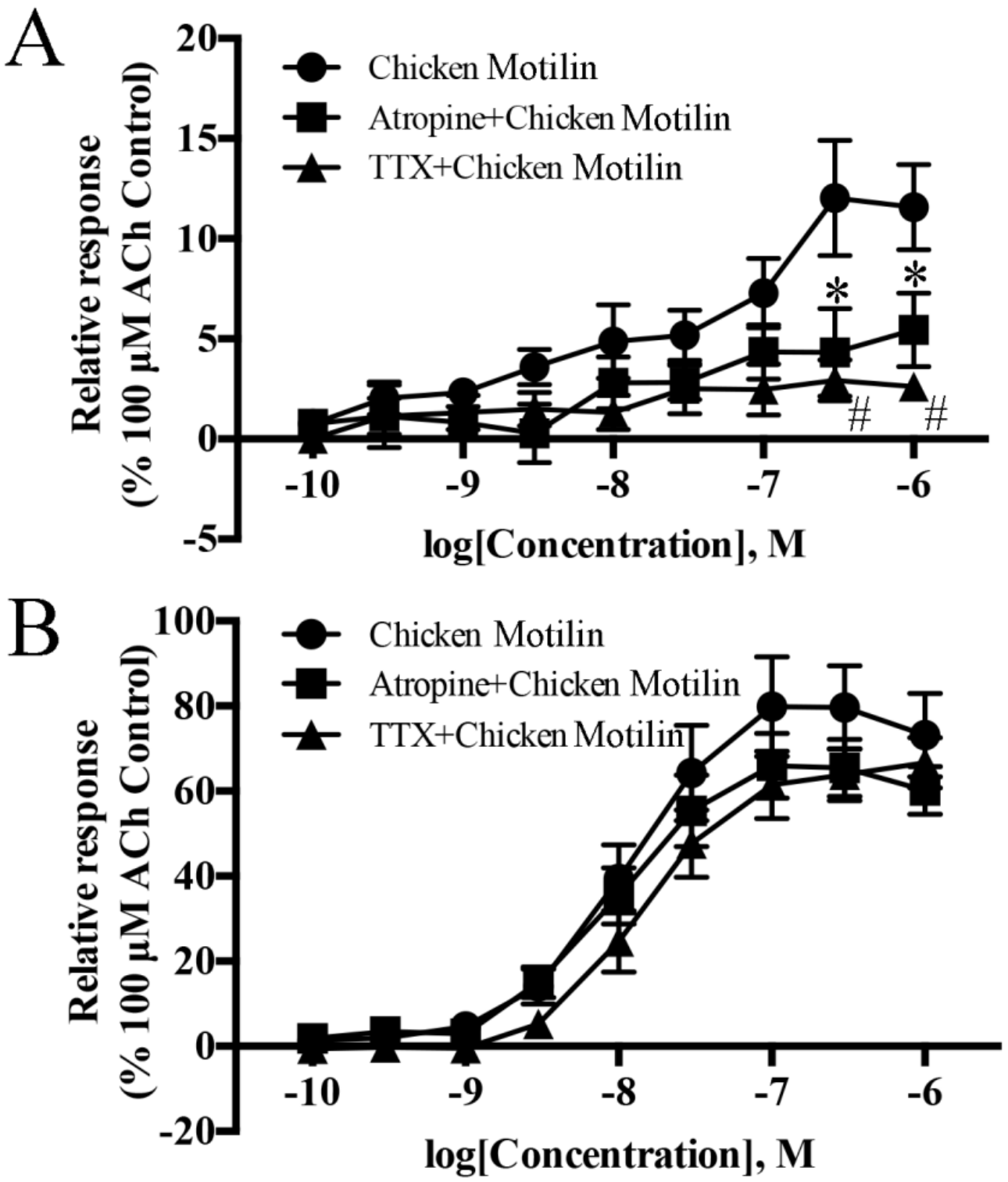

A
Relative response
(% 100 μM ACh Control)
20
15
10
5
0
-5
Chicken Motilin
Atropine+Chicken Motilin
TTX+Chicken Motilin
*
*
#
#
-10
-9
-8
-7
-6
log[Concentration], M
B
Relative response
(% 100 μM ACh Control)
100
80
60
40
20
0
-20
Chicken Motilin
Atropine+Chicken Motilin
TTX+Chicken Motilin
-10
-9
-8
-7
-6
log[Concentration], M

Fig. □

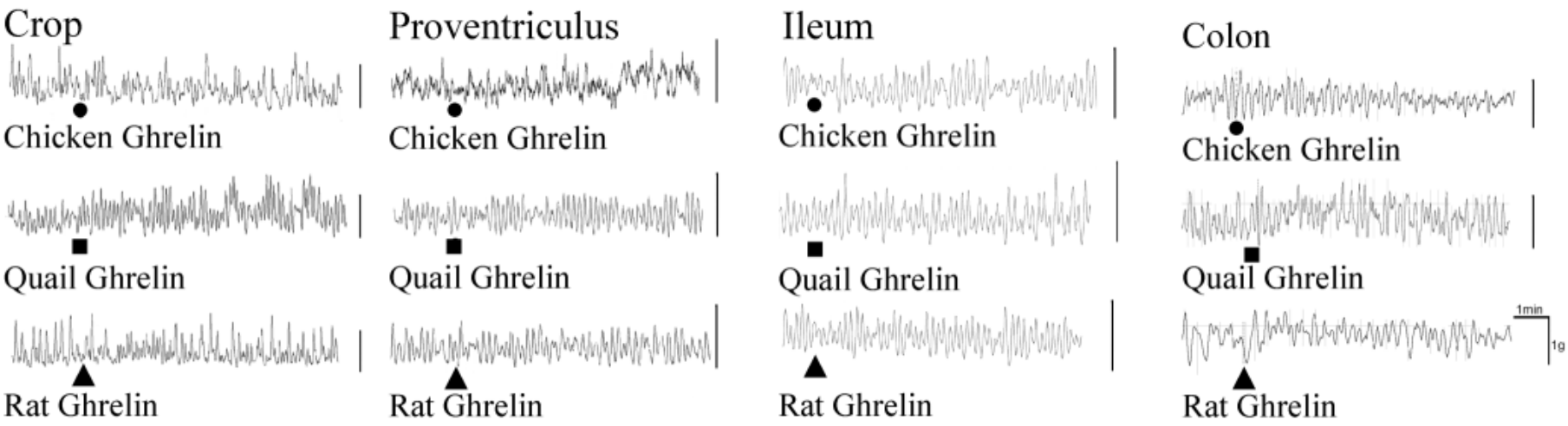

Crop
Chicken Ghrelin
Quail Ghrelin
Rat Ghrelin
Proventriculus
Chicken Ghrelin
Quail Ghrelin
Rat Ghrelin
Ileum
Chicken Ghrelin
Quail Ghrelin
Rat Ghrelin
Colon
Chicken Ghrelin
Quail Ghrelin
Rat Ghrelin
1min
1g

Fig. 9

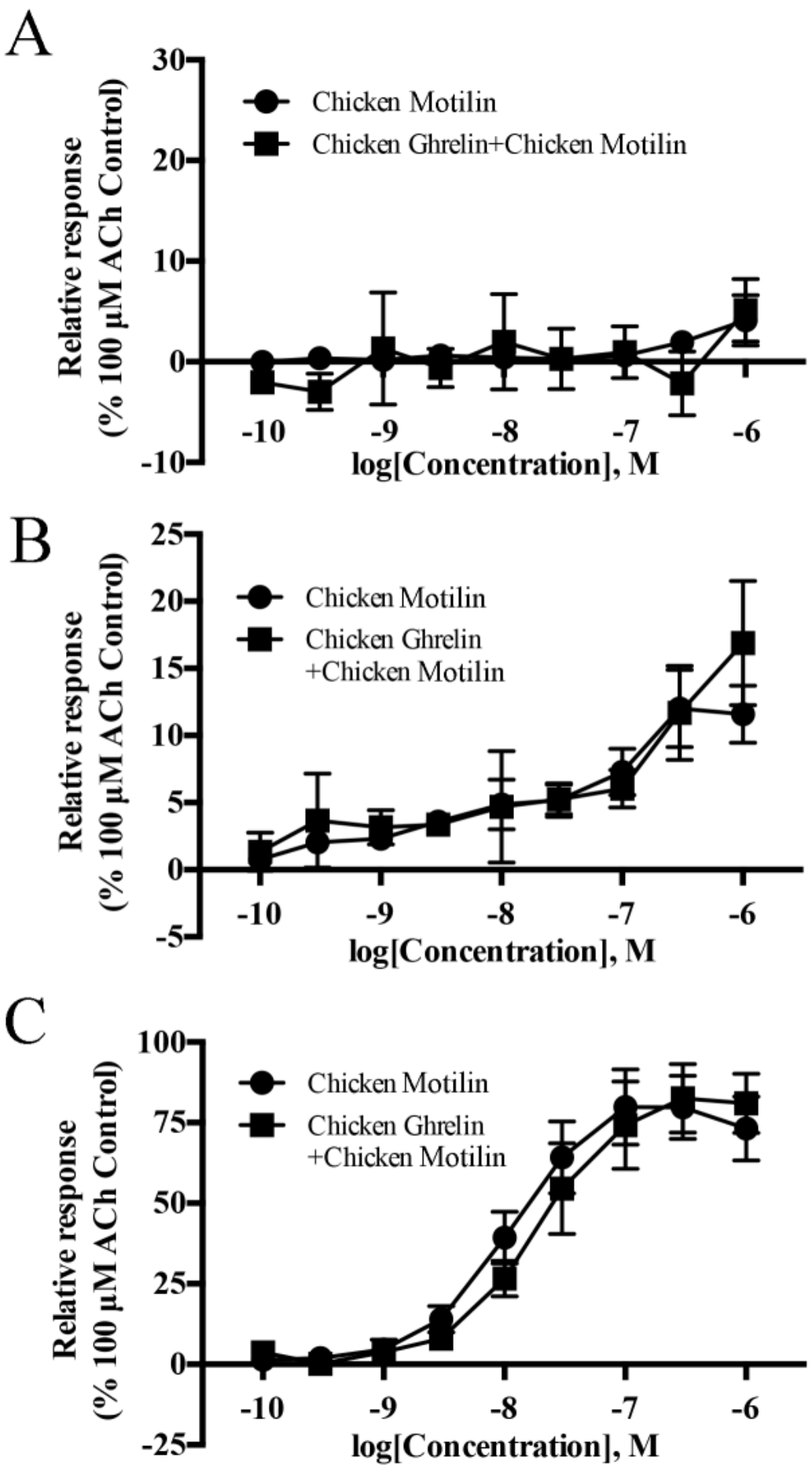
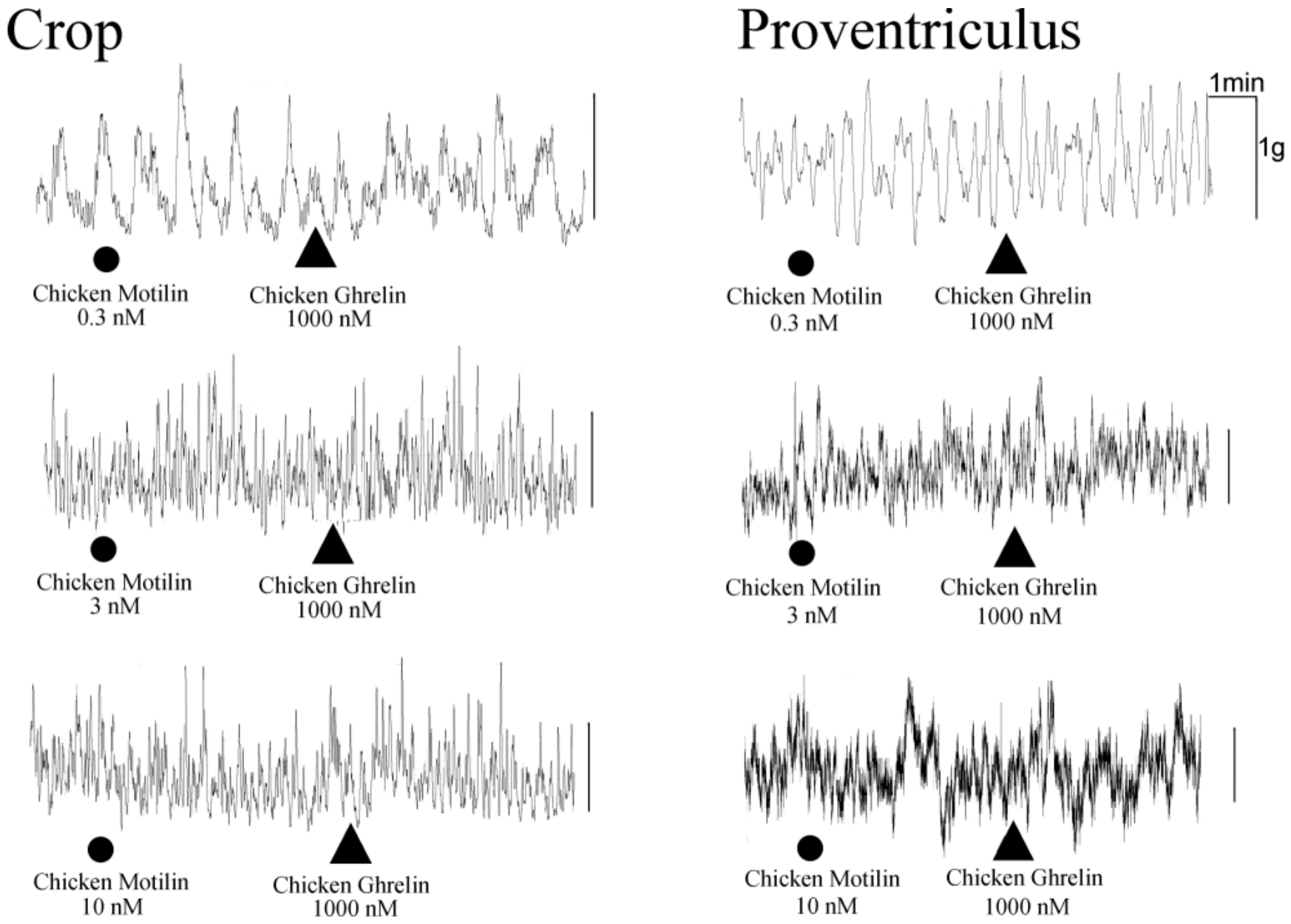

A
Relative response
(% 100 μM ACh Control)
30
20
10
0
-10
Chicken Motilin
Chicken Ghrelin+Chicken Motilin
-10
-9
-8
-7
-6
log[Concentration], M
B
Relative response
(% 100 μM ACh Control)
25
20
15
10
5
0
-5
Chicken Motilin
Chicken Ghrelin
+Chicken Motilin
-10
-9
-8
-7
-6
log[Concentration], M
C
Relative response
(% 100 μM ACh Control)
100
75
50
25
0
-25
Chicken Motilin
Chicken Ghrelin
+Chicken Motilin
-10
-9
-8
-7
-6
log[Concentration], M

Fig.10


Crop
Proventriculus
1min
1g
Chicken Motilin
0.3 nM
Chicken Ghrelin
1000 nM
Chicken Motilin
0.3 nM
Chicken Ghrelin
1000 nM
Chicken Motilin
3 nM
Chicken Ghrelin
1000 nM
Chicken Motilin
3 nM
Chicken Ghrelin
1000 nM
Chicken Motilin
10 nM
Chicken Ghrelin
1000 nM
Chicken Motilin
10 nM
Chicken Ghrelin
1000 nM